IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: GRAVERSEN=1A

P#9

In re Application of:) Art Unit: 1645
)
Jonas GRAVERSEN et al) Examiner:
)
Appln. No.: 09/987,107) Washington, D.C.
)
Filed: November 13, 2001) Confirmation No.: 64[illegible]
)
For: APOLIPOPROTEIN ANALOGUES) February 4, 2002
)
)

REQUEST FOR PRIORITY

Honorable Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with the provisions of 37 CFR §1.55 and the requirements of 35 U.S.C. §119, filed herewith a certified copy of:

Denmark Appln. Nos.: PA 2001 00057 PA 2000 01682	Filed: January 15, 2001 November 10, 2000

It is respectfully requested that applicant be granted the benefit of the priority dates of the foreign application.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By _____
Iver P. Cooper
Registration No. 28,005

IPC:jmb
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528
f:/,h/hoib/graversen1a/PriorityDocPTOCoverLtr4feb02.doc

Kongeriget Danmark

Patent application No.: PA 2001 00057

Date of filing: 15 January 2001

Applicant: ProteoPharma ApS
c/o Østjysk Innovation
Forskerparken, Gustav Wied Vej 10
DK-8000 Århus C

This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

- The specification, claims, figures and sequence listing as filed with the application on the filing date indicated above.

Patent- og Varemærkestyrelsen • Kongeriget Danmark

Patent- og
Varemærkestyrelsen
Erhvervsministeriet

Taastrup, 28 November 2001

Karin Schlichting
Head Clerk

Apolipopr tein analogues

The invention relates to an apolipoprotein construct, a nucleic acid sequence encoding the apolipoprotein construct, a vector comprising the nucleic acid sequence, a method for producing the apolipoprotein construct, a pharmaceutical composition comprising the apolipoprotein construct, and use of the apolipoprotein construct for the preparation of a pharmaceutical composition.

Prior art

In the following, the term Apo A or apolipoprotein A will be used to designate any of the three apolipoproteins, Apolipoprotein A I, Apolipoprotein A II, or Apolipoprotein A III.

Atherosclerosis is the most frequent cause of death in the industrialised countries of the World. One of the pathogenic factors causing atherosclerosis is the deposition of cholesterol in the blood vessels, which ultimately leads to clogging of the vessels.

Apolipoprotein A-1 (apo-A-1) is the main component of plasma HDL (high density lipoprotein), which is negatively correlated to the presence of arterosclerosis. There is strong experimental evidence that this effect is caused by so-called reverse cholesterol transport from peripheral tissues to the liver. There is also experimental evidence that this reverse cholesterol transport can be stimulated in mammals by injection of apo-A-1.

Apolipoprotein A is to a large extent removed from plasma by filtration in the kidneys without being broken down first. The short plasma half-life of apolipoprotein A is a constraint against using the protein in the treatment of atherosclerosis.

US 5,876,968 (SIRTORI ET AL.) concerns substantially pure dimers of a variant of apo-A-1 called apolipoprotein A-1-Milano. Medicaments containing the dimer can be used for preventing thrombosis or they can be used as a prodrug for the monomer. A specific feature of this particular variant of apo-A-I is its ability to form covalent dimers with itself. The authors found that the purified dimer Apo A-I-M/Apo A-I-M has a prolonged plasma half-life compared to the monomer Apo A-I-M.

US 5,643,757 (SHA-IL ET AL.) discloses a method for the production of pure, stable, mature and biologically active human apolipoprotein A-I in high yield.

US 5,990,081 (AGELAND ET AL.) discloses a method for treatment of arterosclerosis or cardiovascular diseases by administering a therapeutically effective amount of apoliproprotein A or apolipoprotein E.

WO 98/37608 (RHONE-POULENC ROHRER ET AL.) describes humane homologous dimers of apolipoprotein A-I variants comprising cystein in position 151. The presence of the cystein residue in the amino acid sequence allows the formation of dimers via disulphide bridges between the monomers. The reference furthermore discloses the corresponding nucleic acid sequences and vectors comprising these as well as pharmaceutical compositions comprising the variants and the use of these in gene therapy.

Summary

In a first aspect the invention relates to an apolipoprotein construct having the general formula

- apo-X,
- where apo is an apolipoprotein component selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, an analogue or a variant thereof,
- and X is a heterologous moiety comprising at least one compound selected from the group consisting of an amino acid, a non-apolipoprotein peptide, an oligomerising module, a carbohydrate, a nucleic acid sequence, a non-apolipoprotein protein, and an apolipoprotein selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, an analogue or a variant thereof,
- with the proviso that when the construct consists of exactly two identical, native apolipoproteins these are linked serially.

Throughout the invention the apolipoprotein component or part of the construct is referred to as apo or apolipoprotein. In the following and in the claims, the

heterologous moiety is referred to as component X of the construct. The apolipoprotein or analogue or variant thereof is linked covalently to the heterologous moiety.

The component X of the construct may be looked broadly upon as a heterologous moiety. In this context a heterologous moiety is any kind of moiety not being linked to apolipoprotein or analogue or variant or functional equivalent thereof under native conditions. The heterologous moiety may thus be a peptide or a protein or part of a peptide or protein from the same or from another species, or even a single amino acid. It may be a synthetic peptide. It may be of carbohydrate nature or of other polymeric and biocompatible nature such as polyols, nucleic acids sequences.

Functional equivalence to native apolipoprotein A-I, A-II or A-IV may conveniently be measured using a lipid binding assay. The ability of the construct to elicit substantially the same physiological response in a mammal may conveniently be measured by measurement of the ability to perform reverse cholesterol transport in a test organism such as rabbits.

The construct comprising apolipoprotein and a heterologous moiety is capable of performing reverse cholesterol transport as well as or even better than native apolipoproteins, despite the modification caused by the addition of a heterologous moiety. The plasma half-life of the construct is preferably increased compared to that of the wild-type apolipoprotein. Preferably the plasma half-life is at least such as doubled or tripled, or at least quadrupled, or at least 10 doubled. Similarly, the binding affinity such as the lipid binding affinity, and/or the cholesterol binding affinity of the construct is preferably increased as compared to wild-type apolipoprotein. Preferably, the lipid binding affinity is increased by at least 5 %, such as at least 10 %, for example at least 15%, such as at least 20%, for example at least 25%, such as at least 30%, for example at least 40% such as at least 50%, for example at least 75%, such as at least 100%, such as at least 150%, for example at least 200%, such as at least 300%.

An increased plasma half-time and/or increased lipid binding affinity have profound implications for the use of the apolipoprotein constructs in the treatment of arteriosclerosis. It is therefore expected that the clinical effect of the apolipoprotein

constructs according to the invention is superior to the effect of wild-type apolipoproteins.

The invention also encompasses analogues or variants of the wild-type apoliproteins capable of eliciting substantially the same physiological response in a mammal.

According to a second aspect of the invention, there is provided a nucleotide sequence encoding an apolipoprotein construct as defined above. Preferably the nucleotide sequence is operably linked to a regulatory sequence for expression of the protein construct.

According to further aspects of the invention, there is provided a vector comprising the nucleotide sequence encoding the apolipoprotein construct and a transformed host cell comprising the nucleotide sequence as defined above.

The apolipoprotein construct according to the invention may be produced by different methods.

According to a first method a transformed host cell is cultured under conditions promoting the expression of a protein construct according to the invention encoded by DNA inserted into a construct, obtaining and recovering the protein construct and optionally further processing the protein construct.

This method is the preferred method when the whole construct is of polypeptide nature and thus can be encoded by one corresponding nucleic acid sequence.

According to a second method the apolipoprotein construct can be manufactured by chemically synthesising the heterologous moiety and subsequently linking it to the apolipoprotein or analogue obtaining an apolipoprotein construct, which is isolated and optionally processed further. This method is the preferred method, when the heterologous moiety is of non-peptide nature. However there may also be conditions under which it is preferred to synthesise the heterologous moiety chemically, when it is of polypeptide nature. Such conditions may be that the heterologous moiety is rather short such as below 20 amino acids.

According to a third method the apolipoprotein construct can be manufactured by culturing a transformed host cell under conditions promoting the expression of an apolipoprotein or an apolipoprotein analogue encoded by a nucleic acid fragment and subsequently covalently linking the apolipoprotein or apolipoprotein analogue to a heterologous moiety obtaining an apolipoprotein construct, isolating the resulting apolipoprotein construct and optionally further processing the construct.

Finally, the apolipoprotein construct may be produced by culturing a transformed host cell under conditions promoting the expression of a protein encoded by a nucleic acid fragment encoding an oligomerising module and subsequently linking said module to at least one apolipoprotein obtaining an apolipoprotein construct.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising the apolipoprotein construct as described above. Preferably the pharmaceutical composition is capable of being administered parenterally such as through injection.

The invention also encompasses the use of an apolipoprotein construct as defined above for the preparation of a pharmaceutical composition. The pharmaceutical composition may further comprise pharmaceutical acceptable excipients, adjuvants, additives, such as lipids, phospholipids, cholesterol, or triglycerides.

The pharmaceutical composition may be administered intravenously, intraarterially, intramusculary, transdermally, pulmonary, subcutaneously, intradermally, intratechally, through the buccal-, anal-, vaginal-, conjunctival-, or intranasal tissue, or by inoculation into tissue, such as tumour tissue, or by an implant, or orally.

Preferably the pharmaceutical composition is used for the treatment and/or prevention of aterosclerose, angina pectoris, claudicatio, or in the treatment of neutralising endotoxins. The apolipoprotein construct as defined above may also be used for gene therapy, wherein the DNA sequence encoding the apolipoprotein construct is used for transfection or infection of at least one cell population.

Detailed description of the invention

In the following the invention will be described in detail with reference to the following figures.

Figure 1 shows the amino acid sequence (in one letter code) of human apolipoprotein A-I.

Figure 2 shows aligned amino acid sequences (in one letter code) for human, macaque, mouse, baboon, pig, and rat apolipoprotein A-IV.

Figure 3: Amino acid sequence of the amino terminal region of tetranectin. Amino acid sequence (in one letter code) from E1 to L51 of tetranectin. Exon 1 comprises residues E1 to D16 and exon 2 residues V17 to V49, respectively. The alpha helix extends beyond L51 to K52 which is the C-terminal amino acid residue in the alpha helix.

Figure 4 shows an alignment of the amino acid sequences of the trimerising structural element of the tetranectin protein family. Amino acid sequences (one letter code) corresponding to residue V17 to K52 comprising exon 2 and the first three residues of exon 3 of human tetranectin; murine tetranectin (Sørensen et al., Gene, 152: 243 -245, 1995); tetranectin homologous protein isolated from reefshark cartilage (Neame and Boynton, 1992,1996); and tetranectin homologous protein isolated from bovine cartilage (Neame and Boynton, database accession number PATCHX:u22298). Residues at a and d positions in the heptad repeats are listed in boldface. The listed consensus sequence of the tetranectin protein family trimerising structural element comprise the residues present at a and d positions in the heptad repeats shown in the figure in addition to the other conserved residues of the region. "hy" denotes an aliphatic hydrophobic residue.

Figure 5 shows the pT7 H6UbiFx Apo A-I plasmid and its corresponding amino acid sequences. The expressed and purified polypeptide (SEQ ID NO 1) consists of amino acids no 25-267 from human Apo A-I.

Figure 6 shows the pT7 H6UbiFx Cys-Apo A-I plasmid and its corresponding amino acid sequences for. The expressed polypeptide (SEQ ID NO 2) consists of a N-terminal cystein residue and the amino acids no 25-267 from human Apo A-I.

Figure 7 shows the pT7H6 Trip-A-Apo A-I - Amp^R plasmid and its corresponding amino acid sequence. The expressed and purified polypeptide (SEQ ID NO 3) consists of the TTSE, a linking sequence, and amino acids no 25-267 from human Apo A-I.

Figure 8 shows the pT7H6 Trip-A-Apo A-I-del 43 - Amp^R plasmid and its corresponding amino acid sequence. The expressed and purified polypeptide (SEQ ID NO 4) consists of the TTSE, a linking sequence, and amino acids no 68-267 from human Apo A-I.

Figure 9 shows the pT7H6FXCysApoAI plasmid and its corresponding amino acid sequence. The expressed and purified polypeptide (SEQ ID NO 2) consists of a N-terminal cystein residue and the amino acids no 25-267 from human Apo A-I.

Detailed description of the invention

The functionality of the constructs according to the invention and of the apo components of the constructs can be measured by a lipid binding assay such as by the DPMC assay described below. Furthermore, the in vivo effect on reverse cholesterol transport may be measured by administration to test animals such as rabbits fed on a cholesterol rich diet such as the method disclosed in Miyazaki et al (Arteriosclerosis, Thrombosis, and Vascular Biology, 1995; 15:1882-1888).

Kinetics of association of the protein construct with dimyristoyl phosphatidylcholine (DMPC)

The ability of the constructs according to the invention to bind to a lipid can conveniently be measured using a well known assay such as the association to dimyristoyl phosphatidylcholine (DPMC).

The assay was conducted as described in (Pownall et al, Biochemistry, 1978, 17: 83-89). Dried DPMC was suspended in 100 mM NaCl, 50 mM Tris-HCl pH 8.0 above its transition temperature at a concentration of 1 mg/ml. The protein sample and the DMPC suspension was both preincubated at room temperature for 10

minutes, and then mixed at a rati of DMPC:protein of 50:1. The reduction in turbidity of the mixture, reflecting increasing lipid-protein association, was followed by measuring the absorbance of the mixture at 325 nm. The turbidity clearance curves were fitted to a double exponential equation, and $t_{1/2}$ were determined.

The apolipoprotein or analogue

In the following the term "apo" is used to designate any protein comprising apolipoprotein A-I, apolipoprotein A-II or apolipoprotein A-IV, any variant or analogue thereof possessing the same lipid binding function.

Preferred apolipoprotein A-I analogues include those disclosed in Figures 5, 6, 7 and 8 and listed as SEQ ID NO 1, 2, 3, and 4.

Known variants of the sequences in Figure 1 include the following variants, indicating the position of the variation with respect to the sequence in Figure 1, the variation, and where appropriate the name of the known variant.

27 P -> H (IN MUNSTER-3C).
27 P -> R.
28 P -> R (IN MUNSTER-3B).
34 R -> L (IN BALTIMORE).
50 G -> R (IN IOWA).
84 L -> R (IN AUTOSOMAL DOMINANT AMYLOIDOSIS).
113 D -> E.
119 A -> D (IN HITA).
127 D -> N (IN MUNSTER-3A).
131 MISSING (IN MARBURG/MUNSTER-2).
131 K -> M.
132 W -> R (IN TSUSHIMA).
134 E -> K (IN FUKUOKA).
160 E -> K (IN NORWAY).
163 E -> G.
167 P -> R (IN GIESSEN).
168 L -> R (IN ZARAGOZA).
171 E -> V.

189 P -> R.

197 R -> C (IN MILANO).

222 E -> K (IN MUNSTER-4).

According to the invention the term "apolipoprotein" is meant to include functional equivalents of at least one sequence in Figure 1 and 2, or a fragment of at least one sequence in Figure 1 and 2, comprising a predetermined amino acid sequence. A "fragment" is defined as:

i) fragments comprising an amino acid sequence capable of being recognised by an antibody also capable of recognising the predetermined amino acid sequences in Figure 1 or 2, and/or

ii) fragments comprising an amino acid sequence capable of binding to a lipid (DPMC assay), which is also capable of binding the predetermined amino acid sequences in Figure 1 or 2.

According to the present invention a functional equivalent of an apolipoprotein or fragments thereof may be obtained by addition, substitution or deletion of at least one amino acid. When the amino acid sequence comprises a substitution of one amino acid for another, such a substitution may be a conservative amino acid substitution. Fragments of the sequences in Figure 1 and 2 may comprise more than one such substitution, such as e.g. two conservative amino acid substitutions, for example three or four conservative amino acid substitutions, such as five or six conservative amino acid substitutions, for example seven or eight conservative amino acid substitutions, such as from 10 to 15 conservative amino acid substitutions, for example from 15 to 25 conservative amino acid substitution, such as from 25 to 75 conservative amino acid substitutions, for example from 75 to 125 conservative amino acid substitutions, such as from 125 to 175 conservative amino acid substitutions. Substitutions can be made within any one or more groups of predetermined amino acids.

Examples of fragments comprising one or more conservative amino acid substitutions including one or more conservative amino acid substitutions within the same group of predetermined amino acids, or a plurality of conservative amino acid

substitutions, wh rein each conservative substitution is generated by substitution within a different group of predetermined amino acids.

Accordingly, a variant of the sequences in Figure 1 or 2, or fragments thereof according to the invention may comprise, within the same variant of the sequences in Figure 1 or 2, or fragments thereof or among different variant of the sequences in Figure 1 or 2, or fragments thereof, at least one substitution, such as a plurality of substitutions introduced independently of one another. Variants of the sequences in Figure 1 or 2, or fragments thereof may thus comprise conservative substitutions independently of one another, wherein at least one glycine (Gly) of said variants of the sequences in Figure 1 or 2, or fragments thereof of the sequences in Figure 1 or 2 is substituted with an amino acid selected from the group of amino acids consisting of Ala, Val, Leu, and Ile, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said alanines (Ala) of said variant of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Val, Leu, and Ile, and independently thereof, variant of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one valine (Val) of said variant of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Leu, and Ile, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said leucines (Leu) of said variant of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Val, and Ile, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one isoleucine (Ile) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Val and Leu, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof wherein at least one of said aspartic acids (Asp) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Glu, Asn, and Gln, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said phenylalanines (Phe) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of

amino acids consisting of Tyr, Trp, His, Pro, and preferably selected from the group of amino acids consisting of Tyr and Trp, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said tyrosines (Tyr) of said variants of the sequences in Figure 1 or 2, or fragments thereof of the sequences in Figure 1 or 2 is substituted with an amino acid selected from the group of amino acids consisting of Phe, Trp, His, Pro, preferably an amino acid selected from the group of amino acids consisting of Phe and Trp, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said arginines (Arg) of said fragment of the sequences in Figure 1 or 2 is substituted with an amino acid selected from the group of amino acids consisting of Lys and His, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one lysine (Lys) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Arg and His, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said aspargines (Asn) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Gln, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one glutamine (Gln) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Asn, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one proline (Pro) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Phe, Tyr, Trp, and His, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said cysteines (Cys) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, and Tyr.

It is clear from the above outline that the same variant or fragment thereof may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

The addition or deletion of an amin acid may be an addition or deletion f from 2 to 10 amino acids, such as from 10 to 20 amino acids, for example from 20 to 30 amino acids, such as from 40 to 50 amino acids. However, additions or deletions of more than 50 amino acids, such as additions from 10 to 200 amino acids, are also comprised within the present invention. More specifically, 43 N-terminal amino acids may be removed from the sequence in Figure 1 without substantially altering the lipid binding effect of the protein. Such a deletion variant is included in SEQ ID NO 4 as the apolipoprotein part of the construct.

It will thus be understood that the invention concerns apolipoproteins comprising at least one fragment of the sequences in Figure 1 or 2 capable of binding lipids such as DPMC, including any variants and functional equivalents of such at least one fragment.

The apolipoprotein according to the present invention, including any functional equivalents and fragments thereof, may in one embodiment comprise less than 243 amino acid residues, such as less than 240 amino acid residues, for example less than 225 amino acid residues, such as less than 200 amino acid residues, for example less than 180 amino acid residues, such as less than 160 amino acid residues, for example less than 150 amino acid residues, such as less than 140 amino acid residues, for example less than 130 amino acid residues, such as less than 120 amino acid residues, for example less than 110 amino acid residues, such as less than 100 amino acid residues, for example less than 90 amino acid residues, such as less than 85 amino acid residues, for example less than 80 amino acid residues, such as less than 75 amino acid residues, for example less than 70 amino acid residues, such as less than 65 amino acid residues, for example less than 60 amino acid residues, such as less than 55 amino acid residues, for example less than 50 amino acid residues.

Fragments

A fragment comprising the lipid binding region of the native sequences in Figure 1 or 2 is particularly preferred. However, the invention is not limited to fragments comprising the lipid binding region. Deletions of such fragments generating functionally equivalent fragments of the sequences in Figure 1 or 2 comprising less

than the lipid binding region are also comprised in the present invention. Functionally equivalent the sequences in Figure 1 or 2 peptides, and fragm nts thereof according to the present invention, may comprise less or more amino acid residues than the lipid binding region. Preferably, the fragment comprises at least the amino acids 100-186 of apo-A-I or a variant or a functional equivalent thereof. It has been determined that this central domain and the α-helices within the domain are directly involved in interactions with phospholipids. Therefore, it is highly likely that this region plays an important role in the functional properties of apo-A-I.

"Functional equivalency" as used in the present invention is according to one preferred embodiment established by means of reference to the corresponding functionality of a predetermined fragment of the sequences in Figure 1 or 2.

Functional equivalents of variants of the sequences in Figure 1 or 2 will be understood to exhibit amino acid sequences gradually differing from the preferred predetermined sequence, as the number and scope of insertions, deletions and substitutions including conservative substitutions increases. This difference is measured as a reduction in homology between the preferred predetermined sequence and the fragment or functional equivalent.

All fragments or functional equivalents of apolipoprotein are included within the scope of this invention, regardless of the degree of homology that they show to a preferred predetermined sequence of apolipoprotein. The reason for this is that some regions of the sequences in Figure 1 or 2 are most likely readily mutatable, or capable of being completely deleted, without any significant effect on the binding activity of the resulting fragment.

A functional variant obtained by substitution may well exhibit some form or degree of native activity of the sequences in Figure 1 or 2, and yet be less homologous, if residues containing functionally similar amino acid side chains are substituted. Functionally similar in this respect refers to dominant characteristics of the side chains such as hydrophobic, basic, neutral or acidic, or the presence or absence of steric bulk. Accordingly, in one embodiment of the invention, the degree of identity between i) a given the sequences in Figure 1 or 2 fragment capable of effect and ii) a preferred predetermined fragment, is not a principal measure of the fragment as a

variant or functional equivalent of a preferred predetermined th sequences in Figure 1 or 2 fragment according to the present invention.

The homology between amino acid sequences may be calculated using well known algorithms such as BLOSUM 30, BLOSUM 40, BLOSUM 45, BLOSUM 50, BLOSUM 55, BLOSUM 60, BLOSUM 62, BLOSUM 65, BLOSUM 70, BLOSUM 75, BLOSUM 80, BLOSUM 85, or BLOSUM 90. Preferably the algorithm BLOSUM 30 is used.

Fragments sharing at least some homology with the sequences in Figure 1 or 2 fragment are to be considered as falling within the scope of the present invention when they are at least about 40 percent homologous with the apolipoprotein or fragment thereof, such as at least about 50 percent homologous, for example at least about 60 percent homologous, such as at least about 70 percent homologous, for example at least about 75 percent homologous, such as at least about 80 percent homologous, for example at least about 85 percent homologous, such as at least about 90 percent homologous, for example at least 92 percent homologous, such as at least 94 percent homologous, for example at least 95 percent homologous, such as at least 96 percent homologous, for example at least 97 percent homologous, such as at least 98 percent homologous, for example at least 99 percent homologous with the sequences in Figure 1 or 2 fragment. According to one embodiment of the invention the homology percentages refer to identity percentages.

Additional factors that may be taken into consideration when determining functional equivalence according to the meaning used herein are i) the ability of antisera against one of the sequences in Figure 1 or 2 to detect fragments of the sequences in Figure 1 or 2 according to the present invention, or ii) the ability of the functionally equivalent fragment to compete with the sequences in Figure 1 or 2 in a lipid binding assay.

Conservative substitutions may be introduced in any position of a preferred predetermined apolipoprotein or fragment thereof. It may however also be desirable to introduce non-conservative substitutions, particularly, but not limited to, a non-conservative substitution in any one or more positions.

A non-conservative substitution leading to the formation of a functionally equivalent fragment of the sequences in Figure 1 or 2 would for example i) differ substantially in polarity, for example a residue with a non-polar side chain (Ala, Leu, Pro, Trp, Val, Ile, Leu, Phe or Met) substituted for a residue with a polar side chain such as Gly, Ser, Thr, Cys, Tyr, Asn, or Gln or a charged amino acid such as Asp, Glu, Arg, or Lys, or substituting a charged or a polar residue for a non-polar one; and/or ii) differ substantially in its effect on polypeptide backbone orientation such as substitution of or for Pro or Gly by another residue; and/or iii) differ substantially in electric charge, for example substitution of a negatively charged residue such as Glu or Asp for a positively charged residue such as Lys, His or Arg (and vice versa); and/or iv) differ substantially in steric bulk, for example substitution of a bulky residue such as His, Trp, Phe or Tyr for one having a minor side chain, e.g. Ala, Gly or Ser (and vice versa).

Substitution of amino acids may in one embodiment be made based upon their hydrophobicity and hydrophilicity values and the relative similarity of the amino acid side-chain substituents, including charge, size, and the like. Exemplary amino acid substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition to the variants described herein, sterically similar variants may be formulated to mimic the key portions of the variant structure and that such compounds may also be used in the same manner as the variants of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

<u>The component X</u>

Preferably, the component X of the protein construct according to the invention is essentially non-immunogenic. For instance the component X may be an amino acid, a carbohydrate, a nucleic acid sequence, an inert protein or polypeptide, which has

substantially n physiological effect and especially no immunological effect on mammals.

Preferably the component X has a long half-life, is non immmunogenic and does not have unwanted effects with regard to ligand binding, i.e. the apolipoprotein component should not be directed at an undesired site through interactions of the X-component with a ligand.

According to one embodiment the component X consists of just one amino acid, which amino acid preferably is a cystein residue, which may be placed N-terminally, C-terminally or internally in the apolipoprotein component.

However, the component X may also comprise a peptide having more than 1 amino acids such as more than 2 amino acids, for example more than 5 amino acids, such as more than 10 amino acids, for example more than 15 amino acids, such as more than 20 amino acids, such as more than 30 amino acids, for example more than 40 amino acids, such as more than 50 amino acids, for example more than 75 amino acids, such as more than 100 amino acids, for example more than 200 amino acids, such as more than 300 amino acids, for example more than 400 amino acids, such as more than 500 amino acids, for example more than 600 amino acids, such as more than 700 amino acids, for example more than 800 amino acids, such as more than 900 amino acids, for example more than 1000, 1250, 1500, 2000, or 2500 amino acids.

One protein could be a plasma protein such as albumin or another non-immunogenic peptide or protein such as the serine protease fragment of plasminogen or another serine protease engineered to be inactive by disruption of the catalytic triad; and the constant region of the heavy chain of immunoglobins. More preferably, the component comprises serum albumin.

According to an especially preferred embodiment of the invention, the component X comprises an apolipoprotein component selected from the group consisting of apolipoprotein A-I, A-II, AIV, an analogue, functional variant or fragment thereof. The two apolipoprotein components may be linked linearly or they may be linked via a non-native cysein bridge. Preferably they are linked linearly.

High r oligomers as well as dimers f the apolipoprotein component comprising at least one non-native cystein residue may be manufactured and linked through cystein bridges under appropriate conditions. Oligomers linked by disulphide bridges may be linked serially (apo-S-S-apo, or apo-S-S-apo-S-S-apo or higher oligomers) or the disulphide bridge may be formed non-terminally by inserting a cystein residue at a non-terminal position in each of the two proteins to be linked and linking the two proteins through a cystin bridge. By inserting two, three or more cystein residues into one or more of the proteins trimers, tetramers and higher multimers may be formed.

The protein construct according to the invention may also comprise two, three or more apolipoproteins or analogues thereof being serially and covalently linked to one another. This may be achieved by linking the C-terminal of a first apolipoprotein to the N-terminus of the next apolipoprotein and so forth. The proteins may be so linked after transcription and translation or the nucleotide sequence may simply comprise two, three or more sequences coding for the apolipoprotein construct in question as well as optional linker peptides between the apolipoproteins.

Thereby, the need for a heterologous moiety to perform the linkage is avoided. It is expected that in the constructs having two, three or more apo units essentially all the apo units will participate in lipid binding thereby contributing to the functionality of the construct. Therefore it is expected that these multi-apo constructs may have an increased lipid binding ability compared to native apo. An additional advantage of these constructs compared to native apo, is that they have an increased plasma half-life compared to native apo.

Such constructs comprising more than one apolipoprotein component may comprise a combination selected from the following group:

Dimers:
A-I A-I; A-II AII; A-IV AIV; A-I A-II; A-I A-IV; A-II A-IV.

Trimers:

A-I A-II A-IV; A-I A-I A-II; A-I A-I A-I; A-I A-I A-IV; A-II A-II A-I; A-II A-II A-IV; A-II A-II A-II; A-IV A-IV A-IV; A-IV A-IV A-II; A-IV A-IV A-I.

Oligomerisation modules

According to an especially preferred embodiment of the invention, the heterologous moiety is an oligomerising module. In this context, an oligomerising module is a peptide or a protein or part of a protein which is capable of interacting with other, similar or identical oligomerising modules. The interaction is of the type that produces multimeric proteins or polypeptides. Such an interaction may be caused by covalent bonds between the components of the multimer as well as by hydrogen bond forces, hydrophobic forces, van der Waals forces, salt bridges. The invention also encompasses oligomerising modules of non-peptide nature such as a nucleic acid sequence of DNA, RNA, LNA, or PNA.

The oligomerisation module may be a dimerising module, a trimerising module, a tetramerising module, or a multimerising module.

When the apolipoprotein or analogue part of the construct is coupled to an oligomerising module, multimers of the construct can be made by simply mixing a solution of constructs (oligomerisation module linked to apolipoprotein part) under appropriate conditions. In this way, dimers, trimers, tretramers, pentamers, hexamers or higher –mers can be made depending on the type of oligomerising module being linked to the apolipoprotein part of the construct.

The multimers according to the invention may be homomers or heteromers, since different apolipoproteins can be linked to the oligomerising modules and be incorporated into the multimer. It may be advantageous to mix the different types of apolipoproteins in this way to obtain an improved clinical effect of the construct. Preferred homomers include trimers of Apo-A-I and trimers of Apo-A-IV.

According to an especially preferred embodiment of the invention the oligomerising module is from tetranectin and more specifically comprises the tetranectin trimerising structural element (hereafter termed TTSE), which is described in detail in WO 98/56906. The trimerising effect of TTSE is caused by a coiled coil structure

which interacts with the coiled coil structure of two other TTSEs to form a trimer, which is exceptionally stable. A further advantage of TTSE is that it is a weak antigen (WO 98/56906).

Preferably the heparin binding site, which is located in the N-terminal region of exon 1 (Figure 4) is abolished by removal or mutagenis of N-terminal lysine residues (Graversen et al., manuscript) without inhibiting trimerisation. TTSEs that include most or all of exon 1 therefore confer an affinity for sulfated polysaccharides to any designed protein which encompasses such a TTSE as part of its structure. If desired, however, this affinity can be reduced or abolished by N-terminal truncation or mutagenesis of lysine residues in the part of the TTSE that corresponds to the N-terminal 8-10 amino acid residues of exon 1 (Graversen et al., unpublished).

The interacting domain of the trimerising module according to the invention is preferably of the same type as in TTSE, namely a triple alpha helical coiled coil.

The TTSE may be from human tetranectin, from rabbit tetranectin, from murine tetranectin or from C-type lectin of shark cartilage. Preferably, the TTSE comprises a sequence having at least 68%, such as at least 75%, for example at least 81%, for example at least 87% such as at least 92% identity with the consensus sequence of figure 1. Thereby analogues of the TTSE having substantially the same trimerising effect are encompassed by the invention.

Preferably, the cystein residue 50 of TTSE should be mutagenised to serine, threonine, methionine or to any other amino acid residue in order to avoid formation of an unwanted inter-chain disulphide bridge, which could lead to unwanted multimerisation.

The presence of a trimer may be ascertained by well known techniques such as gel-filtration, SDS-PAGE, or native SDS gel electrophoresis depending on the nature of the trimer. One preferred method for ascertaining the presence of an oligomer is through linkage by DMSI followed by SDS-PAGE.

According to a preferred embodiment of the invention the protein construct is obtained by linking two or more apolipoproteins to oligomerising modules. The advantage of this embodiment is that the linkage of the individual apolipoproteins to

ne another does not take place within the apolipoprotein but in the oligomerising module. Thereby the nature of the wild-type apolipoprotein is conserved and the apolipoprotein conserves the secondary and tertiary structure, which is advantageous for its physiological function. By further introducing a peptide spacer between the apolipoprotein and the oligomerising module it is ensured that both of the components of the construct can perform their interaction with lipids and other oligomerising modules respectively without being affected by the interactions of the other component. Preferably, the peptide spacer is non-immunogenic, and has an essentially linear three dimensional structure.

Different or identical apo units may be oligomerised using an oligomerisation module such as a dimerising module, a trimerising module, a tetramerising module, a pentamerising module, a hexamerising module or a multimerising module. The oligomerising modules may comprise a coiled coil structure capable of interchain recognition and interaction.

The general method for producing an artificial oligomer of a protein or peptide comprises the identification of a trimerisation module from proteins that form trimers in nature. Through careful analysis, the domain responsible for the protein-protein interaction can be identified, isolated, and linked to the protein or peptide to be trimerised. According to the invention such trimerisation does not necessarily comprise the formation of a trimer of apolipoprotein or an analogue. It is also possible to link just one apolipoprotein to a trimerisation module and allow this peptide to trimerise with two other trimerisation modules. Thereby the molecular weight of the apolipoprotein part is increased and the plama half-life may be increased compared to native apolipoprotein.

One example of an oligomerisation module is disclosed in WO 95/31540 (HOPPE ET AL.), which describes polypeptides comprising a collectin neck region. The amino acid sequence constituting the collectin neck region may be attached to any polypeptide of choice. Trimers can then be made under appropriate conditions between three polypeptides comprising the collectin neck region amino acid sequence.

Spacer peptide

Th protein construct may also advantageously comprise a spacer moiety, which is covalently linked between the apolipoprotein or apolipoprotein analogue and the heterologous moiety. The effect of the spacer is to provide space between the heterologous moiety and the apolipoprotein part of the construct. Thereby is ensured that the secondary structure of the apolipoprotein part is not affected by the presence of the heterologous moiety so that the physiological effect of the apolipoprotein part is maintained. Preferably, the spacer is of polypeptide nature. In this way the nucleic acid sequence encoding the spacer can be linked to the sequence encoding the apolipoprotein part of the construct and optionally the sequence for the heterologous moiety, and the whole construct can be produced at the same time.

Design and preparation of suitable spacer moieties are known in the art and are conveniently effected by preparing fusion polypeptides having the format apo-spacer-X, where the spacer moiety is a polypeptide fragment (often a relatively inert one), so as to avoid undesired reactions between the spacer and the surroundings or the construct.

A spacer moiety may also be inserted between two TTSEs allowing both of these to interact with a third separate TTSE to form a trimeric complex, which then comprises two separate peptides: TTSE and TTSE-spacer-TTSE. This embodiment facilitates the production of the apolipoprotein construct since the major part of the trimer, which is then strictly seen a dimer, can be synthesised as one single polypeptide comprising in sequence (apo denoting any polypeptide sequence forming the apolipoprotein part of the construct) apo-TTSE-spacer-TTSE-apo.

In the embodiments where two TTSEs are present in the same monomer it is preferred that the spacer moiety has a length and a conformation which favours complex formation involving both of the two TTSEs which are covalently linked by the spacer moiety. In this way, problems arising from undesired formation of trimers of the formats (2+1+1), (2+2+2), and (2+2+1) (wherein only one TTSE of each monomer participates in complex formation) can be diminished.

The spacer peptide preferably comprises at least two amino acids, such as at l ast three amino acids, for example at least five amino acids, such as at least ten amino acids, for example at least 15 amino acids, such as at least 20 amino acids, for example at least 30 amino acids, such as at least 40 amino acids, for example at least 50 amino acids, such as at least 60 amino acids, for example at least 70 amino acids, such as at least 80 amino acids, such as at least 90 amino acids such as approximately 100 amino acids.

The spacer may be linked to the apo component and X through covalent linkages. and preferably the spacer is essentially non-immunogenic, and/or is not prone to proteolytic cleavage, and/or does not comprise any cystein residues.

Similarly, the three-dimensional structure of the spacer is preferably linear or substantially linear.

The following are examples of spacer sequences, which are believed to be especially preferable for linking apolipoprotein analogues to a component X. Preferred examples of spacer or linker peptides include those, which have been used to link proteins without substantially impairing the function of the linked proteins or at least without substantially impairing the function of one of the linked proteins. More preferably the linkers or spacers have been used to link proteins comprising coiled-coil structures.

Tetranectin based linker:

The linker may include the tetranectin residues 53-56, which in tetranectin forms a β-strand, and the residues 57-59 which forms a turn in tetranectin (Nielsen BB, Kastrup JS, Rasmussen H, Holtet TL, Graversen JH, Etzerodt M, Thøgersen HC, Larsen IK, FEBS-Letter 412, 388-396, 1997). The sequence of the segment is GTKVHMK. This linker has the advantage that it in native tetranectin is bridging the trimerisation domain with the CRD-domain, and hence is imagined to be well suited for connecting the trimerisation domain to another domain in general. Furthermore the resulting construct is not expected to be more immunogenic than the construct without a linker. The tetranectin based linker is highly preferred when the component X comprises the TTSE.

Fibronectin based linker:

The linker may be chosen as a sub-sequence from the connecting strand 3 from human fibronectin, this corresponds to amino acid residues 1992-2102 (SWISS-PROT numbering, entry P02751). Preferably the subsequence: PGTSGQQPSVGQQ covering amino acid residues number 2037-2049 is used, and within that subsequence the segment GTSGQ corresponding to amino acid residues 2038-2042 is more preferable. This construct has the advantage that it is know not to be highly prone to proteolytic cleavage and is not expected to be highly immunogenic bearing in mind that fibronectin is present at high concentrations in plasma.

Human IgG_3 upper hinge based linker

The 10 amino acid residue sequence derived from the upper hinge region of murine IgG_3, PKPSTPPGSS, has been used for the production of antibodies dimerised trough a coiled coil (Pack P. and Plückthun, A. Biochemistry **31**, pp 1579-1584 (1992)) and may be useful as a spacer peptide according to the present invention. Even more preferable may be a corresponding sequence from the upper hinge region of human IgG_3. Sequences from human IgG_3 are not expected to be immunogenic in human beings.

Flexible linkers

Possible examples of flexible linker/spacer sequences include SGGTSGSTSGTGST, AGSSTGSSTGPGSTT or GGSGGAP. These sequences have been used for the linking of designed coiled coils to other protein domains (Müller, K. M., Arndt, K. M. and Alber, T., Meth. Enzymology, **328**, pp 261-281 (2000).

<u>The linkage</u>

The two components of the construct may be linked together by a covalent linkage. This linkage may be formed between the component X and the C or N terminal amino acid of the apo component. The components may also be linked via more than one covalent linkages. The covalent linkage between the components may also comprise a S-S bridge, preferably between cystein residues. These cystein residues

may be placed C or N terminally in the apo component and/or the component X or the may be placed internally in either or both of the components.

Carbohydrate

Irrespective of the other components of the construct the construct according to the invention may comprise a carbohydrate moiety.

Tetranectin trimerising structural element

One especially preferred embodiment of the invention is the trimerisation or partial trimerisation of an apolipoprotein or analogue thereof with the trimerisation module from tetranectin.

This technique is described in WO 98/56908 (THØGERSEN ET AL.), which is hereby incorporated by reference. The trimeric polypeptides are constructed as a monomer polypeptide construct comprising at least one tetranectin trimerising structural element (TTSE), which is covalently linked to at least one heterologous moiety. The tetranectin trimerising structural element is capable of forming a stable complex with two other tetranectin trimerising structural elements.

The term "trimerising structural element" (TTSE) used in the present description and claims is intended to refer to the portion of a polypeptide molecule of the tetranectin family which is responsible for trimerisation between monomers of the tetranectin polypeptide. The term is also intended to embrace variants of a TTSE of a naturally occurring tetranectin family member, variants which have been modified in the amino acid sequence without adversely affecting, to any substantial degree, the trimerisation properties relative to those of the native tetranectin family member molecule.

Specific examples of such variants will be described in detail herein, but it is generally preferred that the TTSE is derived from human tetranectin, murine tetranectin, C-type lectin of human or bovine cartilage, or C-type lectin of shark cartilage. Especially preferred is monomer polypeptide constructs including at least one TTSE derived from human tetranectin.

The 49 residue polypeptide sequence encoded by exons 1 and 2 of tetranectin (Fig. 3) appears to be unique to the tetranectin group of proteins (Fig. 4) as no significant sequence homology to other known polypeptide sequences has been established. In preparation for experimental investigations of the architecture of tetranectin a collection of recombinant proteins was produced, the collection including complete tetranectin, the CRD domain (approximately corresponding to the polypeptide encoded by exon 3), a product corresponding to the polypeptide encoded by exons 2+3, a product corresponding to exons 1+2 (Holtet et al., 1996). Tetranectin is indeed a trimer, but the exon 2 encoded polypeptide is in fact capable of effecting trimerisation by itself as evidenced by the observation that the recombinant protein corresponding to exons 2+3 is in fact trimeric in solution.

3D-structure analysis of crystals of full-length recombinant tetranectin (Nielsen et al., 1996; Nielsen, 1996; Larsen et al., 1996; Kastrup, 1996) has shown that the polypeptide encoded in exon 2 plus three residues encoded in exon 3 form a triple alpha helical coiled coil structure.

From the combination of sequence and structure data it becomes clear that trimerisation in tetranectin is in fact generated by a structural element (Fig. 4), comprising the amino acid residues encoded by exon two and the first three residues of exon 3 by an unusual heptad repeat sequence, that apparently is unique to tetranectin and other members of its group: This amino acid sequence (Fig. 4) is characterised by two copies of heptad repeats (abcdefg) with hydrophobic residues at a and d positions as are other alpha helical coiled coils. These two heptad repeats are in sequence followed by an unusual third copy of the heptad repeat, where glutamine 44 and glutamine 47 not only substitute the hydrophobic residues at both the a and d position, but are directly involved in the formation of the triple alpha helical coiled coil structure. These heptad repeats are additionally flanked by two half-repeats with hydrophobic residues at the d and a position, respectively.

The presence of beta-branched hydrophobic residues at a or d positions in alpha helical coiled coil are known to influence the state of oligomerisation. In the tetranectin structural element only one conserved valine (number 37) is present. At sequence position 29 in tetranectin no particular aliphatic residue appears to be

preferred.

In summary, it is apparent that the triple stranded coiled coil structure in tetranectin to a large extent is governed by interactions that are unexpected in relation to those characteristic among the group of known coiled coil proteins.

The TTSEs form surprisingly stable trimeric molecules. The experimental observations, that (1) a substantial part of the recombinant proteins exists in the oligomeric state of and can be cross-linked as trimeric molecules even at 70C° and (2) that exchange of monomers between different trimers can only be detected after exposure to elevated temperature are evidence of a extremely high stability of the tetranectin trimerising structural element. This feature must be reflected in the amino acid sequence of the structural element. In particular, the presence and position of the glutamine containing repeat in the sequential array of heptad repeats is, together with the presence and relative position of the other conserved residues in the consensus sequence (Fig. 4), considered important for the formation of these stable trimeric molecules. For most practical uses the cysteine residue 50 should be mutagenized to serine, threonine, methionine or to any other amino acid residue in order to avoid formation of an unwanted inter-chain disulphide bridge, which eventually would lead to uncontrolled multimerisation, aggregation and precipitation of a polypeptide product harbouring this sequence.

In particular in conjunction with the trimer-stabilising exon 1 encoded polypeptide, the tetranectin trimerising structural element is a truly autonomous polypeptide module retaining its structural integrity and propensity to generate a highly stable homotrimeric complex whether it is attached or not by a peptide bond at either or at both termini to other proteins.

This unique property is demonstrated by the fact that polypeptide sequences derived from heterologous proteins may readily be trimerised when joined as fusion proteins to the tetranectin trimerising structural element. This remains valid irrespective of whether the heterologous polypeptide sequences are placed amino-terminally or carboxy-terminally to the trimerising element allowing for the formation of one molecular assembly containing up to six copies of one particular polypeptide sequence or functional entities, or the formation of one molecular assembly

containing up to six different polypeptide sequences, each contributing their individual functional property.

Since three TTSEs of naturally occurring human tetranectin forms up a triple alpha helical coiled coil, it is preferred that the stable complex formed by the TTSEs of the invention also forms a triple alpha helical coiled coil.

The "tetranectin family" are polypeptides, which share the consensus sequence shown in Fig. 4 or a sequence, which is homologous at sequence level with this consensus sequence.

Hence, monomer polypeptide constructs of the invention are preferred which comprise a polypeptide sequence which has at least 68% sequence identity with the consensus sequence shown in Fig. 4, but higher sequence identities are preferred, such as at least 75%, at least 81%, at least 87%, and at least 92%.

Production of the protein construct

In order to produce a peptide component of the protein construct the cDNA encoding this part is inserted into an expression vector and transformed into a host cell.

The above mentioned host cell (which is also a part of the invention) can be prepared by traditional genetic engineering techniques which comprises inserting a nucleic acid fragment (normally a DNA fragment) encoding the polypeptide part of a monomer polypeptide construct of the invention into a suitable expression vector, transforming a suitable host cell with the vector, and culturing the host cell under conditions allowing expression of the polypeptide part of the monomer polypeptide construct. The nucleic acid fragment encoding the polypeptide may be placed under the control of a suitable promoter which may be inducible or a constitutive promoter.

Depending on the expression system, the polypeptide may be recovered from the extracellular phase, the periplasm or from the cytoplasm of the host cell.

Suitable vector systems and host cells are well-known in the art as evidenced by the

vast amount of literature and materials available to the skilled person. Since the present invention also relates to the use of the nucleic acid fragments of the invention in the construction of vectors and in host cells, the following provides a general discussion relating to such use and the particular considerations in practising this aspect of the invention.

In general, of course, prokaryotes are preferred for the initial cloning of nucleic sequences of the invention and constructing the vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example, strains such as E. coli K12 strain 294 (ATCC No. 31446), E. coli B, and E. coli X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes are also preferred for expression, since efficient purification and protein refolding strategies are available. The aforementioned strains, as well as E. coli W3110 (F-λ, prototrophic, ATCC No. 273325), bacilli such as Bacillus subtilis, or other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various Pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.

The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system

(Goeddel et al., 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilised, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in E. coli from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. Saccharomyces cerevisiase, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980).

This plasmid already contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expres sion vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase,

and enzymes responsible for maltose and galactos utilisation. Any plasmid vector containing a yeast compatibl promoter, origin f replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate in culture (tissue culture) has become a routine procedure (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the HindIII site toward the BglI site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilise promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Upon production of the polypeptide monomer constructs it may be necessary to process the polypeptides further, e.g. by introducing non-proteinaceous functions in the polypeptide, by subjecting the material to suitable refolding conditions (e.g. by using the generally applicable strategies suggested in WO 94/18227), or by cleaving off undesired peptide moieties of the monomer (e.g. expression enhancing peptide fragments which are undesired in the end product).

In the light of the above discussion, the methods for recombinantly producing the monomer polypeptide construct of the invention are also a part of the invention, as are the vectors carrying and/or being capable of replicating the nucleic acids according to the invention in a host cell or a cell-line. According to the invention the expression vector can be e.g. a plasmid, a cosmid, a minichromosome, or a phage. Especially interesting are vectors which are integrated in the host cell/cell line genome after introduction in the host.

Another part of the invention are transformed cells (useful in the above-described methods) carrying and capable of replicating the nucleic acid fragments of the invention; the host cell can be a microorganism such as a bacterium, a yeast, or a protozoan, or a cell derived from a multicellular organism such as a fungus, an insect cell, a plant cell, or a mammalian cell. Especially interesting are cells from the bacterial species Escherichia, Bacillus and Salmonella, and a preferred bacterium is E. coli.

Yet another part of the invention relates to a stable cell line producing the polypeptide part of a construct according to the invention, and preferably the cell line carries and expresses a nucleic acid of the invention.

Plasmids

The construct according to the invention may be manufactures using the plasmids disclosed below.

<u>pT7H6 TripA-apoAI:</u>

The plasmid comprises the plasmid pT7H6Fxtripa described in WO 98/56906 as example no. 1.

Expression is governed by the T7 promoter. The plasmid furthermore comprises a H6 sequence being a hexa-His affinity tag for use in purification. After that is inserted a Fxa recognition sequence (IQGR).

-SPGT is a connective sequence to the subsequent trimerisation module. This sequence has been inserted because it gives the opportunity to cut the DNA strand with Bgl II and Kpn I.

–Trip A is the trimerisation module from tetranectin..

GS is another connective sequence, which provides an opportunity to cut with Bam. HI.

Finally the plasmid comprises the human apoliprotein A-I cDNA coding for amino acids 25 -287 from human apolipoprotein A-I

pT7H6TripA-apoA1-del43:

The plasmid comprises the sequences as above, but the apolipoprotein part has been replaced with cDNA coding for amino acids 68-267 from human apolipoprotein A-I.

pT7H6UbiFxApoAI

The basic plasmid has been described in Ellgaard et al (1997).

The plasmid comprises the following sequences:

- the expression is governed by the T7 promoter

-H6: hexa-His affinity tag for purification of the protein construct

-Ubi: cDNA coding for human ubiquitin

-FX: recognition sequence for Fxa

-DNA coding for two Gly residues, necessary for the optimal cleavage by FXa.

-ApoAI: cDNA coding for amino acids 25 -267 from human apolipoprotein A-I

pT7H6UbiFXCysApoAI

As above, but after the sequence coding for the two glycine residues and before the apolipoprotein A-I sequence coding for a cystein residue has been inserted.

PT7H6FXCysApoAI

The plasmid comprises the following sequences:

- the expression is governed by the T7 promoter
-H6: hexa-His affinity tag for purification of the protein construct
-FX: recognition sequence for Fxa
-DNA coding for two Gly residues, necessary for the optimal cleavage by FXa.
-DNA coding for a cystein residue.
-ApoAI: cDNA coding for amino acids 25 -267 from human apolipoprotein A-I

Receptor binding

The performance of the constructs according to the invention may be analysed by measuring the ability of the constructs to bind to receptors which bind native apolipoprotein A-I, A-II or A-IV. Such receptors include but are not limited to cubilin and scavenger receptor B1. The dissociation constant, KD, of the complex between cubilin and native apolipoprotein A I is 20 nm. It has been determined experimentally that an apolipoprotein A I trimer according to the present invention binds even stronger to cubilin.

Affinity tags

The protein construct according to the invention may also comprise an affinity tag for use during purification of the construct. Such a tag preferably comprises a polyhistidine sequence. This sequence can advantageously be used for purification of the product on a Ni^{2+} column, which will bind the polyhistidine sequence and thereby the whole protein. After elution from the column the polyhistidine sequence may be cleaved off by a proteinase such as trombin recognising a specific sequence built into the construct between the protein construct and the polyhistidine sequence.

In order to aid in the purification of the expressed protein a DNA sequence encoding an affinity tag may be added to the sequence encoding the protein construct. Such

affinity tags may include well known tags as an antigenic tag, a polyhistidine tag, or a GST tag.

Production of apo-TTSE

In order to produce a construct comprising an apolipoprotein part and a TTSE, the cDNA encoding the apolipoprotein part is ligated at the 3' end to the 5' end of the c-DNA encoding the TTSE. Further TTSE units and apolipoprotein units may also be ligated. A sequence encoding an enzyme cleavage site is further ligated to the 3' end of the sequence encoding TTSE and finally a sequence encoding polyhistidine is also ligated. This can be done by conventional PCR techniques. The combined c-DNA is inserted into an expression vector and transformed into a host cell.

After expression in the E. coli, the polyhistidine sequence is used to capture the heterologous protein on a Ni2+ column. After elution the polyhistidine tail can be removed by a proteinase such as Fx cleaving the heterologous protein at the specific site inserted into it between the TTSE and the polyhistidine sequence. The resulting apo-TTSE peptide can then be processed further by trimerising it to other or identical apo-TTSE peptides.

Use of an apo construct for preparation of a pharmaceutical composition

The apo construct may be used for the preparation of a pharmaceutical composition. The composition may comprise pharmaceutical acceptable excipients, adjuvants, additives such as phospholipids, cholesterol, or triglycerides.

The pharmaceutical composition may be administered intravenously, intraarterially, intramusculary, transdermally, pulmonary, subcutaneously, intradermally, intratechally, through the buccal-, anal-, vaginal-, conjunctival-, or intranasal tissue, or by inoculation into tissue, such as tumour tissue, or by an implant, or orally.

The formulation of the pharmaceutical compositions according to the invention is preferably performed using techniques well known to the skilled practitioner. This may comprise the addition of pharmaceutically acceptable excipients, adjuvants, or additives, such as phospholipids, cholestorol or triglycerides.

Administration of apo construct

The apo-constructs according to the invention may be administered for arteriosclerosis such as for indications such as angina pectoris, claudication and for removal of endotoxins. It is envisaged that the administration comprises the administration of at least 50 mg of the construct every week such as to obtain a plasma concentration of approximately 0.5 g/L. Preferably the construct is administered parenterally such as through injections, suppositories, implants etc.

Preferably the composition is administered in an amount comprising at least 50 mg apolipoprotein construct per week, such as at least 100 mg/week, for example at least 250 mg/week, such as at least 500 mg/week, for example at least 750 mg/week such as at least 1000 mg/week, for example at least 1250 mg/week, such as at least 1500 mg/week, for example at least 2000 mg/week, such as at least 2500 mg/week, for example at least 5000 mg/week. The administration may be performed once a week once every second week, or once every third week, or once every fourth week.

The constructs may also be administered orally. For this administration route, the technology described in WO9946283, US 5,922,680, US 5,780,434 or US 5,591,433, US 5,609,871, or US 5,783,193 may be applied to the protein constructs according to the present invention. These references are hereby incorporated in their entirety by reference.

Cell population:

The invention also encompasses the use of the nucleotide sequence according to the invention for gene therapy.

The genes may be transferred to a population of macrophages and subsequently be transferred to the patient in need of treatment. Hereby, a transient expression of the gene is obtained, since the macrophage have a limited lifetime in the blood vessels.

Permanent transfection may be obtained by transforming liver cells.

Example 1: Cloning of Apo A-I

The cDNA encoding Apo A-I was amplified from a human liver cDNA library (Clontech) using standard PCR techniques. For the construction of Ubi-A-I the primers used were: 5´-CAC GGA TCC ATC GAG GGT AGG GGT GGA GAT GAA CCC CCC CAG AGC-3´ and 5´- TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG-3´. The product was cloned into the vector pT7H6Ubi, described in (Ellgaard L. et al Eur. J. Biochem. 1997;244(2):544-51) using the Bam HI and Hind III cloning sites. For the construction of Trip-A-A-I the primers used were 5´-AAG GGA TCC GAT GAA CCC CCC CAG AGC CCC-3´ and 5´-TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG-3´. The PCR product was cloned into the pT7H6tripa vector described in WO 98/56906 using the Bam HI and Hind III cloning sites. For the construction of Trip-A-I-del43 the primers used were 5´-AGG GGA TCC CTA AAG CTC CTT GAC AAC TGG G-3´ and 5´- TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG -3´. The PCR product was cloned into the pT7H6tripa vector described in WO 98/56906 using the Bam HI and Hind III cloning sites. For the construction of Ubi-Cys-A-I the primers used were: 5´-GGT GGA TCC ATC GAG GGT AGG GGT GGA TGT GAT GAA CCC CCC C -3´ and 5´- TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG -3´. The product was cloned inti the vector pT7H6Ubi, described in (Ellgaard L. et al Eur. J. Biochem. 1997;244(2):544-51) using the Bam HI and Hind III cloning sites. The plasmids generated are shown on figure 4, 5, 6, and 7.

Example 2: Expression of apolipoprotein A-I (apo A-I) in *E. coli*

Ubi-A-I and Trip-A-I are conveniently expressed in *E. coli* AV-1 cells (Stratagene Inc.). Other cell lines may be used as well. Culturing of the cells and induction of expression were performed as described for tetranectin in WO 98/56906.

Example 3: Isolation and processing of protein

Crude protein was isolated by phenol extraction as described for tetranectin in WO 98/56906. The re-dissolved pellet from 6 litres of expression culture was centrifuged to remove non-dissolved material and then batch adsorbed to 50 ml Ni^{2+}-NTA-

Sepharose, prepared as described in WO 98/56906. The column material was packed on a column and then washed with 500 ml 8 M urea, 500 mM NaCl, 50 mM Tris-HCl pH 8.0, then 200 ml of 6 M Guanidinium-HCl, 50 mM Tris-HCl pH 8.0 and finally 300 ml of 500 mM NaCl, 50 mM Tris-HCl pH 8.0. The protein was eluted with 500 mM NaCl, 50 mM Tris-HCl pH 8.0 and 10 mM EDTA. The protein was added 0.5 mg of FX_a and digested overnight at room temperature. Thrombin may be used for this purpose as well. The protein was gelfiltrated on a G-25 sephadex (Pharmacia) column in to a 500 mM NaCl, 50 mM Tris-HCl pH 8.0 buffer. Undigested protein was removed by passing the protein solution over a Ni^{2+}-NTA-Sepharose column pre-washed in 500 mM NaCl, 50 mM Tris-HCl pH 8.0 and then washed with 500 mM NaCl, 50 mM Tris-HCl pH 8.0. Undigested protein was eluted with 500 mM NaCl, 50 mM Tris-HCl pH 8.0 and 10 mM EDTA.

Example 4: Removal of lipids from the proteins

The proteins were gelfiltrated into a 10 mM $(NH_4)_2CO_3$ pH 8.8 solution and lyophilised. The lyophilised protein was resuspended in 25 ml cold 1:1 methanol/chloroform, incubated on ice for 30 min, centrifuged at 3000 *g* for 20 minutes. The pellet was resuspended in 25 ml of 1:2 cold methanol/chloroform, equilibrated for 30 minutes on ice and recentrifuged. The supernatant was removed and the pellet was briefly air-dried and then redissolved in 6 M guanidinium-HCl, 50 mM Tris-HCl pH 8.0 over night.

Example 5: Multimerisation assay

Cross linking

Multimerisation may be measured by cross-linking of multimers followed by analytical SDS-PAGE.

60 µl of a 0.2 mg/ml protein dissolved in 150 mM Na-borate pH 9.0 equillibrated to the desired temperature for 30 minutes are added 5 µl of a 20 mg/ml dimethylsuberimidate and incubated for 30 minutes at the desired temperature. The cross-linking was quenched by the addition of 5 µl 3 M Tris-HCl pH 9.0.

Dimethylsuberimidate causes lysin residues located within a short distance from one another to form a covalent bond. The result is that proteins which have formed multimers are covalently linked to one another. The molecular weight of the multimers can be estimated in the subsequent SDS-PAGE.

The cross-linking products were analysed by SDS-PAGE on 8-16 % acrylamide gels. Optionally an adjuvant, such as a lipid, was included in the cross-linking mixture, in which case the protein was pre-incubated with the adjuvant.

Analytical gelfiltration

Multimerisation may also be measured by analytical gelfiltration.

The protein was dissolved in a 500 mM NaCl, 50 mM Tris-HCl pH 8.0 buffer and gelfiltrated on a Superdex 200 HR 10/30 column in to the desired buffer at room temperature and a flow of 0.25 ml/min. For standard procedures the buffer was 100 mM NaCl, 50 mM Tris-HCl pH 8.0.

REFERENCES

Bergeron et al 1997, Biochem Biophys Acta, 1344:139-152.

Bolivar et al, 1977. Gene, 2: 95.

Chang et al. 1978. Nature, 275: 617-624.

Ellgaard et al (1997). Dissection of the domain architecture of the $\alpha_2$macroglobulin-receptor-associated protein. Eur J Biochem vol 244:544-551.

Fiers et al. 1978. Nature, 273: 113.

Goeddel et al. 1979. Nature, 281: 544.

Hess et al. 1969. Advances in Enzyme Regulation, 7: 149-168.

Hitzman et al. 1980. Journal of Biological Chemistry, 25: 12073-12080.

Holland et al. 1978. Biochemistry, 17: 4900.

Holtet, T.L., Graversen, J.H., Thøgersen, H.C. and Etzerodt, M. (1996).Domains and shared motifs in plasminogen - ligand interaction. Poster 21st Annual Lorne Conference on Protein Structure and Function, held Melbourne, Australia, February 4-8, 1996.

Itakura et al. 1977. Science, 198: 1056.

Jones. 1977. Genetics, 85: 23-33.

Kastrup, J.S. (1998). Lecture at Minisymposium held by EU HCM contract CHRX-CT93-0143: Protein Crystallography I in Hamburg, Germany, December 13-14, 1998.

Kingsman et al, 1979, Gene :141.

Larsen, I.K., Nielsen, B.B., Rasmussen, H. and Kastrup, J.S. (1996). P ster, 17th International Crystallography Congress, Seattle, USA held August 8-17. 1996.

Neame, P.J. and Boynton, R.E. (1996). Protein Soc. Symposium, (Meeting date 1995; 9th Meeting: Tech. Prot. Chem VII). Proceedings pp. 401-407 (Ed., Marshak, D.R.; Publisher: Academic, San Diego, Calif.).

Nielsen, B.B. (1996). Lecture, Lundbeck Centre Neuro-Medicinal Chemistry Minisymposium held November 5, 1996 at the Royal Danish School of Pharmacy, Copenhagen.

Nielsen, B.B., Larsen, I.K., Rasmussen, H. and Kastrup, J.S. (1996). Lecture, Danish Crystallographer's Meeting, held June 3-4, 1996 at the Royal Danish School of Pharmacy, Copenhagen.

Siebwenlist et al. 1980. Cell, 20: 269.

Sørensen et al, 1995, Gene, 152:243-245.

Stinchomb et al. 1979. Nature 282: 39.

Tschemper et al. 1980. Gene, 10: 157.

Claims

1. An apolipoprotein construct having the general formula

- apo-X,

- where apo is an apolipoprotein component selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, an analogue or a variant thereof,

- and X is a heterologous moiety comprising at least one compound selected from the group consisting of an amino acid, a non-apolipoprotein peptide, an oligomerising module, a carbohydrate, a nucleic acid sequence, a non-apolipoprotein protein, and an apolipoprotein selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, apolipoprotein E, an analogue or a variant thereof,

- with the proviso that when the construct consists of exactly two identical, native apolipoproteins these are linked serially.

2. The construct of claim 1, further comprising a spacer between the apo component and X.

3. The construct of claim 2, wherein the spacer comprises a spacer peptide.

4. The construct of claim 3, wherein the spacer peptide comprises at least two amino acids, such as at least three amino acids, for example at least five amino acids, such as at least ten amino acids, for example at least 15 amino acids, such as at least 20 amino acids, for example at least 30 amino acids, such as at least 40 amino acids, for example at least 50 amino acids, such as at least 60 amino acids, for example at least 70 amino acids, such as at least 80 amino acids, such as at least 90 amino acids such as approximately 100 amino acids.

5. The construct according to claim 2, wherein the spacer is linked to the apo component and X through covalent linkages.

6. The construct according to claim 2, wherein the spacer is essentially non-immunogenic, and/or is not prone to proteolytic cleavage and/or does not comprise any cystein residues.

7. The construct according to claim 2, wherein the three-dimensional structure of the spacer is linear or substantially linear.

8. The construct according to claim 2, wherein the spacer peptide comprises the amino acid sequence GTKVHMK from tetranectin, amino acid sequence PGTSGQQPSVGQQ and GTSGQ from the connecting strand 3 from human fibronectin, PKPSTPPGSS from the upper hinge region of murine IgG_3, SGGTSGSTSGTGST, AGSSTGSSTGPGSTT or GGSGGAP.

9. The construct of claim 1, wherein the component X is linked by a covalent link to the N-terminal or the C-terminal amino acid of apo.

10. The construct of claim 1, wherein the apo component and X are linked by two covalent links.

11. The construct of claim 1, wherein the apo component and X are linked through a S-S bridge, preferably a cystin bridge.

12. The construct of claim 1, wherein the protein comprises a non-immunogenic inert protein.

13. The construct of claim 12, wherein the protein comprises a protein selected from the group comprising albumin, more preferably serum albumin, the serine protease fragment of plasminogen or another serine protease engineered to be inactive by disruption of the catalytic triad, and the constant region of the heavy chain of immunoglobulins.

14. The construct of claim 1, wherein the analogue or variant is capable of eliciting substantially the same physiological response as the apolipoprotein-A-I, A-II or A-IV.

15. The construct of claim 1, wherein the amino acid constituting the component X is a cystein residue.

16. The construct of claim 15, wherein the cystein residue is linked N-terminally, C-terminally, or internally to the apo component.

17. The construct of claim 1, wherein the peptide constituting the component X comprises more than 1 amino acids such as more than 2 amino acids, for example more than 5 amino acids, such as more than 10 amino acids, for example more than 15 amino acids, such as more than 20 amino acids, such as more than 30 amino acids, for example more than 40 amino acids, such as more than 50 amino acids, for example more than 75 amino acids, such as more than 100 amino acids, for example more than 200 amino acids, such as more than 300 amino acids, for example more than 400 amino acids, such as more than 500 amino acids, for example more than 600 amino acids, such as more than 700 amino acids, for example more than 800 amino acids, such as more than 900 amino acids, for example more than 1000, 1250, 1500, 2000, or 2500 amino acids..

18. The construct of claim 1, wherein the protein comprised in the component X comprises two proteins selected from the group consisting of apolipoprotein A-I, apolipoprotein A-II, or apolipoprotein A-IV, an analogue or variant thereof.

19. The construct of claim 1, wherein the protein comprised in the component X comprises at least one apolipoprotein E.

20. The construct of claim 1, wherein the oligomerising module is a dimerising module.

21. The construct of claim 1, wherein the oligomerising module is a trimerising module.

22. The construct of claim 1, wherein the oligomerising module is a tetramerising modul.

23. The construct f claim 1, wherein the oligomerising module is a multimerising module.

24. The construct of claim 1, wherein the oligomerising module is of non-peptide nature.

25. The construct of claim 21, wherein the trimerising module comprises an amino acid sequence, capable of mediating interchain recognition, trimerisation and alignment of three polypeptide chains.

26. The construct of claim 21, wherein the trimerising module is from tetranectin.

27. The construct of claim 21, wherein the trimerising module comprises the tetranectin trimerising module from tetranectin.

28. The construct of claim 25, wherein the tetranectin trimerising module is capable of forming a stable complex with other tetranectin trimerising modules.

29. The construct of the claims 28, wherein the stable complex includes a coiled coil structure.

30. The construct of claim 29, wherein the coiled coil structure is a triple alpha helical coiled coil.

31. The construct of the claims 25 to 30, wherein the trimerising module comprising two tetranectin trimerising modules linked by a spacer moiety, which allows both of the two tetranectin trimerising modules to take part in a complex formation with a third tetranectin trimerising module not being part of the apolipoprotein construct.

32. The construct of the claims 25 to 31, wherein at least one tetranectin trimerising module is selected from the group consisting of human tetranectin, murine tetranectin or C-type lectin of human, bovine or shark cartilage.

33. The construct of the claims 25 to 32, wherein the tetranectin trimerising module comprises a sequence having at least 68 % identity with the consensus sequence of Fig. 4.

34. The construct of claim 33, wherein the sequence identity with the consensus sequence is at least 75 %, such as at least 81 %, for example at least 87 %, such as at least 92 %.

35. The construct of claim 33, wherein the cystein residue no. 50 is substituted by a serine residue, a threonine residue, or a methionine residue.

36. The construct of claim 33, wherein the cystein residue no. 50 is substituted by any other amino acid residue.

37. The construct of claim 21, comprising the trimerisation module from the collectin neck region.

38. The construct of claim 1, wherein the oligomerising module is present after purification of the apolipoprotein construct.

39. The construct of claim 1, further comprising at least one carbohydrate moiety.

40. The construct of claim 1, further comprising an affinity tag.

41. The construct of claim 40, comprising a polyHis affinity tag.

42. The construct of claim 40, comprising an affinity tag selected from the group consisting of an antigenic tag, a GST tag.

43. The construct of claim 1, having a half-life of at least the half-life of native Apo A-I, A-II or A-IV, preferably at least 2 times higher, more preferably at least 3 times higher such as 4 times, more preferably at least 5 times higher, such as 6 times, more preferably at least 8 times higher such as at least 10 times.

44. The construct f claim 1, having a higher binding affinity to cholesterol compared to native Apo A-I, A-II or A-IV.

45. The construct of claim 1, capable of binding to cubilin.

46. The construct of claim 1, capable of binding to the scavenger receptor type B1.

47. The construct of claim 1, causing substantially no immunoresponse in humans.

48. The construct of claim 1, wherein the nucleic acid sequence comprises a DNA, a RNA, a PNA, or a LNA sequence.

49. Nucleic acid comprising a sequence of nucleotides encoding an apolipoprotein construct as defined in claim 1 to 48.

50. The nucleic acid of claim 49, wherein the encoding sequence is operably linked to a regulatory sequence for expression of the protein construct.

51. A vector comprising the nucleic acid of the claims 49 or 50.

52. A transformed host cell, comprising a nucleic acid sequence as defined in the claims 49 or 50.

53. A method for the production of an apolipoprotein construct as defined in the claims 1-48, comprising the steps of:

- culturing a transformed host cell under conditions promoting the expression of a protein construct according to claims 1 to 48,
- obtaining and recovering said protein construct,
- optionally, further processing said protein construct.

54. A method for the production of an apolipoprotein construct as defined in the claims 1-48, comprising the steps of:

- chemically synthesizing at least one oligomerising module, and subsequently
- linking said module to at least one apolipoprotein, an apolipoprotein analogue or an apolipoprotein variant,
- obtaining an apolipoprotein construct,
- isolating the resulting apolipoprotein construct,
- optionally, further processing said construct.

55. A method for the production of an apolipoprotein construct as defined in the claims 1-48, comprising the steps of:

- culturing a transformed host cell under conditions promoting the expression of an apolipoprotein or an apolipoprotein analogue or an apolipoprotein variant encoded by a nucleic acid fragment, and subsequently

- covalently linking said apolipoprotein or apolipoprotein analogue or apolipoprotein variant to a heterologous moiety,

- obtaining an apolipoprotein construct,

- isolating the resulting apolipoprotein construct,

- optionally, further processing said construct.

56. A method for the production of an apolipoprotein construct as defined in the claims 1-48, comprising the steps of:

- culturing a transformed host cell under conditions promoting the expression of a oligomerising module encoded by a nucleic acid fragment, and subsequently

- covalent linking said module to at least one apolipoprotein, an apolipoprotein analogue or an apolipoprotein variant,

- obtaining an apolipoprotein construct,

- isolating the resulting apolipoprotein construct,

- optionally, further processing said construct.

57. A pharmaceutical composition, comprising the apolipoprotein construct as defined in the claims 1-48.

58. The pharmaceutical composition of claim 57 adapted to be injected.

59. Use of an apolipoprotein construct as defined in the claims 1-48.

60. Use of an apolipoprotein construct as defined in the claims 1 to 48 for the preparation of a pharmaceutical composition.

61. The use of claim 60, wherein the pharmaceutical composition further comprises pharmaceutical acceptable excipients, adjuvants, additives, such as phospholipids, cholesterol, or triglycerides.

62. The use of claim 60, wherein the pharmaceutical composition is administered intravenously, intraarterially, intramusculary, transdermally, pulmonary, subcutaneously, intradermally, intratechally, through the buccal-, anal-, vaginal-, conjunctival-, or intranasal tissue, or by inoculation into tissue, such as tumour tissue, or by an implant, or orally.

63. The use of claim 60, comprising administering to an individual a composition comprising at least 50 mg apolipoprotein construct per week.

64. The use of claim 60, comprising administering to an individual a composition comprising at least at least 100 mg/week, for example at least 250 mg/week, such as at least 500 mg/week, for example at least 750 mg/week such as at least 1000 mg/week, for example at least 1250 mg/week, such as at least 1500 mg/week, for example at least 2000 mg/week, such as at least 2500 mg/week, for example at least 5000 mg/week.

65. The use of claim 60, comprising administering a dose of a pharmaceutical composition once a week.

66. The use of claim 60, comprising administering a dose of a pharmaceutical composition once every second week, or once every third week, or once every fourth week.

67. The use of claim 60, for the treatment and/or prevention of aterosclerosis.

68. The use of claim 60, in the treatment of neutralising end toxins.

69. The use of claim 60, in the treatment of angina pectoris.

70. The use of claim 60, in the treatment of claudicatio.

71. Use of the nucleic acid sequence as defined in the claims 49 to 50 for gene therapy, wherein the DNA sequence encoding said apolipoprotein construct is used for transfection or infection of at least one cell population.

72. The use of claim 71, wherein the at least one cell population comprises macrophages.

73. The use of claim 71, wherein the at least one cell population comprises liver cells.

FIGURE 1

SQ SEQUENCE 267 AA; 30778 MW; 1A28B8366E620310 CRC64;

MKAAVLTLAV LFLTGSQARH FWQQDEPPQS PWDRVKDLAT VYVDVLKDSG RDYVSQFEGS
ALGKQLNLKL LDNWDSVTST FSKLREQLGP VTQEFWDNLE KETEGLRQEM SKDLEEVKAK
VQPYLDDFQK KWQEEMELYR QKVEPLRAEL QEGARQKLHE LQEKLSPLGE EMRDRARAHV
DALRTHLAPY SDELRQRLAA RLEALKENGG ARLAEYHAKA TEHLSTLSEK AKPALEDLRQ
GLLPVLESFK VSFLSALEEY TKKLNTQ

FIGURE 2

```
sp|P06727|APA4_HUMAN      MFLKAVVLTLALVAVAGARAEVSADQVATVMWDYFSQLSNNAKEAVEHLQKSELTQQLNA 60
sp|P33621|APA4_MACFA      MFLKAVVLTLALVAVTGARAEVSADQVATVMWDYFSQLSSNAKEAVEHLQKSELTQQLNA 60
sp|P06728|APA4_MOUSE      MFLKAAVLTLALVAITGTRAEVTSDQVANVVWDYFTQLSNNAKEAVEQFQKTDVTQQLST 60
sp|Q28758|APA4_PAPAN      ----------------GARAEVSADQVATVMWDYFSQLSSNAKEAVEHLQKSELTQQLNA 44
sp|O46409|APA4_PIG        MFLKAVVLSLALVAVTGARAEVNADQVATVMWDYFSQLGSNAKKAVEHLQKSELTQQLNT 60
sp|P02651|APA4_RAT        MFLKAVVLTVALVAITGTQAEVTSDQVANVMWDYFTQLSNNAKEAVEQLQKTDVTQQLNT 60
                                  *:::***.:****.*:****:**..***:***::**:::****.:

sp|P06727|APA4_HUMAN      LFQDKLGEVNTYAGDLQKKLVPFATELHERLAKDSEKLKEEIGKELEELRARLLPHANEV 120
sp|P33621|APA4_MACFA      LFQDKLGEVNTYAGDLQKKLVPFATELHERLAKDSEKLKEEIRKELEEVRARLLPHANEV 120
sp|P06728|APA4_MOUSE      LFQDKLGDASTYADGVHNKLVPFVVQLSGHLAKETERVKEEIKKELEDLRDRMMPHANKV 120
sp|Q28758|APA4_PAPAN      LFQDKLGEVNTYAGDLQKKLVPFATELHERLAKDSKKLKEEIRKELEEVRARLLPHANEV 104
sp|O46409|APA4_PIG        LFQDKLGEVNTYTEDLQKKLVPFATELHERLTKDSEKLKEEIRRELEELRARLLPHATEV 120
sp|P02651|APA4_RAT        LFQDKLGNINTYADDLQNKLVPFAVQLSGHLTKETERVREEIQKELEDLRANMMPHANKV 120
                          ********; .**: .:::*****..:*  :*:*::::::*** :***::* .::***.:*

sp|P06727|APA4_HUMAN      SQKIGDNLRELQQRLEPYADQLRTQVNTQAEQLRRQLTPYAQRMERVLRENADSLQASLR 180
sp|P33621|APA4_MACFA      SQKIGENVRELQQRLEPYTDQLRTQVNTQTEQLRRQLTPYAQRMERVLRENADSLQTSLR 180
sp|P06728|APA4_MOUSE      TQTFGENMQKLQEHLKPYAVDLQDQINTQTQEMKLQLTPYIQRMQTTIKENVDNLHTSMM 180
sp|Q28758|APA4_PAPAN      SQKIGENVRELQQRLEPYTDQLRTQVNTQTEQLRRQLTPYAQRMERVLRENADSLQTSLR 164
sp|O46409|APA4_PIG        SQKIGDNVRELQQRLGPFTGGLRTQVNTQVQQLQRQLKPYAERMESVLRQNIRNLEASVA 180
sp|P02651|APA4_RAT        SQMFGDNVQKLQEHLRPYATDLQAQINAQTQDMKRQLTPYIQRMQTTIQDNVENLQSSMV 180
                          :* :*:*:::**::* *:: *: *:*:*.:::: **.** :**: .:::*  .*.:*:

sp|P06727|APA4_HUMAN      PHADELKAKIDQNVEELKGRLTPYADEFKVKIDQTVEELRRSLAPYAQDTQEKLNHQLEG 240
sp|P33621|APA4_MACFA      PHADQLKAKIDQNVEELKERLTPYADEFKVKIDQTVEELRRSLAPYAQDAQEKLNHQLEG 240
sp|P06728|APA4_MOUSE      PLATNLKDKFNRNMEELKGHLTPRANELKATIDQNLEDLRRSLAPLTVGVQEKLNHQMEG 240
sp|Q28758|APA4_PAPAN      PHADQLKAKIDQNVEELKGRLTPYADEFKVKIDQTVEELRRSLAPYAQDAQEKLNHQLEG 224
sp|O46409|APA4_PIG        PYADEFKAKIDQNVEELKGSLTPYAEELKAKIDQNVEELRRSLAPYAQDVQEKLNHQLEG 240
sp|P02651|APA4_RAT        PFANELKEKFNQNMEGLKGQLTPRANELKATIDQNLEDLRSRLAPLAEGVQEKLNHQMEG 240
                          * * ::* *:::*:* **  *** *:*:*..***.:*:**  *** : ..*******:**

sp|P06727|APA4_HUMAN      LTFQMKKNAEELKARISASAEELRQRLAPLAEDVRGNLKGNTEGLQKSLAELGGHLDQQV 300
sp|P33621|APA4_MACFA      LAFQMKKNAEELKARISASAEELRQRLAPLAEDMRGNLRGNTEGLQKSLAELGGHLDRHV 300
sp|P06728|APA4_MOUSE      LAFQMKKNAEELQTKVSAKIDQLQKNLAPLVEDVQSKVKGNTEGLQKSLEDLNRQLEQQV 300
sp|Q28758|APA4_PAPAN      LAFQMKKNAEELKARISASAEELRQRLAPLAEDMRGNLRGNTEGLQKSLAELGGHLDRHV 284
sp|O46409|APA4_PIG        LAFQMKKQAEELKAKISANADELRQKLVPVAENVHGHLKGNTEGLQKSLLELRSHLDQQV 300
sp|P02651|APA4_RAT        LAFQMKKNAEELQTKVSTNIDQLQKNLAPLVEDVQSKLKGNTEGLQKSLEDLNKQLDQQV 300
                          *:*****:****:::::*:. ::*::.*.*:.*:.*:::.:::********** :*  :*:::*

sp|P06727|APA4_HUMAN      EEFRRRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDKVNSFFSTFKE 360
sp|P33621|APA4_MACFA      EEFRLRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDKVNSFFSTFKE 360
sp|P06728|APA4_MOUSE      EEFRRTVEPMGEMFNKALVQQLEQFRQQLGPNSGEVESHLSFLEKSLREKVNSFMSTLEK 360
sp|Q28758|APA4_PAPAN      EEFRLRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDKVNSFFSTFKE 344
sp|O46409|APA4_PIG        EEFRLKVEPYGETFNKALVQQVEDLRQKLGPLAGDVEGHLSFLEKDLRDKVNTFFSTLKE 360
sp|P02651|APA4_RAT        EVFRRAVEPLGDKFNMALVQQMEKFRQQLGSDSGDVESHLSFLEKNLREKVSSFMSTLQK 360
                          * **  *** *: ** *****:*.:**:**. :*:**.*******.**:**.:*:**:::

sp|P06727|APA4_HUMAN      KESQDKTLSLPEL----EQQQEQQQ----------------------------------EQ 383
sp|P33621|APA4_MACFA      KESQDNTLSLPEP----EQQREQQQEQQQEQEQEQQQQQEQQQQQEQQREQQQQEQQQEQ 416
sp|P06728|APA4_MOUSE      KGSPDQPQALPLPEQAQEQAQEQAQEQVQ------------------------------- 389
sp|Q28758|APA4_PAPAN      KESQDNTLSLPEP----EQQQEQQQEQEQ-----QQEQQEEQQQQEQQ-------QEQEQ 388
sp|O46409|APA4_PIG        EASQGQSQALPAQ----EKAQ---------------------------------------- 377
sp|P02651|APA4_RAT        KGSPDQPLALPLP----EQVQEQVQEQVQ------------------------------- 385
                          : * .:. :**       *: :

sp|P06727|APA4_HUMAN      QQEQVQMLAPLES 396
sp|P33621|APA4_MACFA      QQEQVQMLAPLES 429
sp|P06728|APA4_MOUSE      -------PKPLES 395
sp|Q28758|APA4_PAPAN      QQEQVQMLAPLES 401
sp|O46409|APA4_PIG        --------APLEG 382
sp|P02651|APA4_RAT        -------PKPLES 391
                                 ***.
```

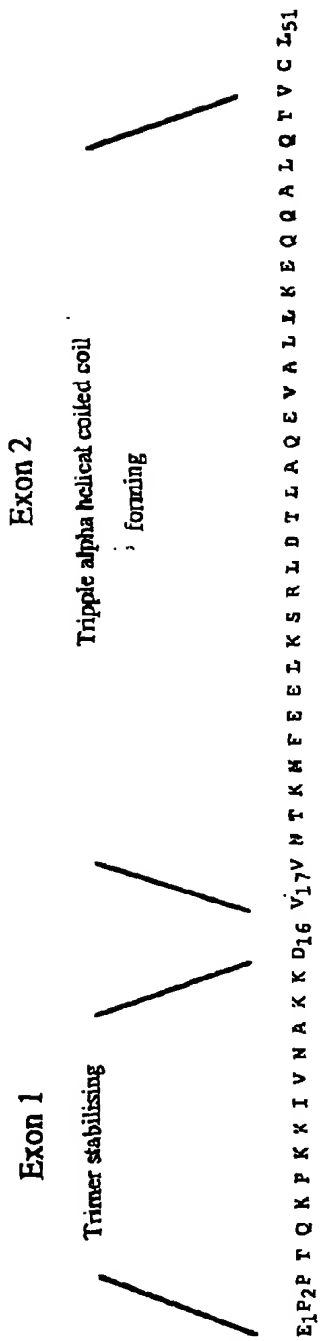

Exon 1
Trimer stabilising
Exon 2
Trippie alpha helical coiled coil
forming
E1P2P T Q K P K K I V N A K K D16 V17V N T K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T V C L51

Figure 3

Position	d e f g a b c d e f g a b c d e f g a
Human tetranectin	V V N T K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T V C L K
Murine tetranectin	L V S S K M F E E L K N R M D V L A Q E V A L L K E K Q A L Q T V C L K
Bovine cart. protein	R R V K E K D G D L K T Q V E K L W R E V N A L K E M Q A L Q T V C L R
Shark cart. protein	S K S G K G K D D L R N E I D K L W R E V N S L K E H Q A L Q T V C L K
Consensus	L hy L E V L K E Q A L Q T V C L

Figure 4

FIGURE 5

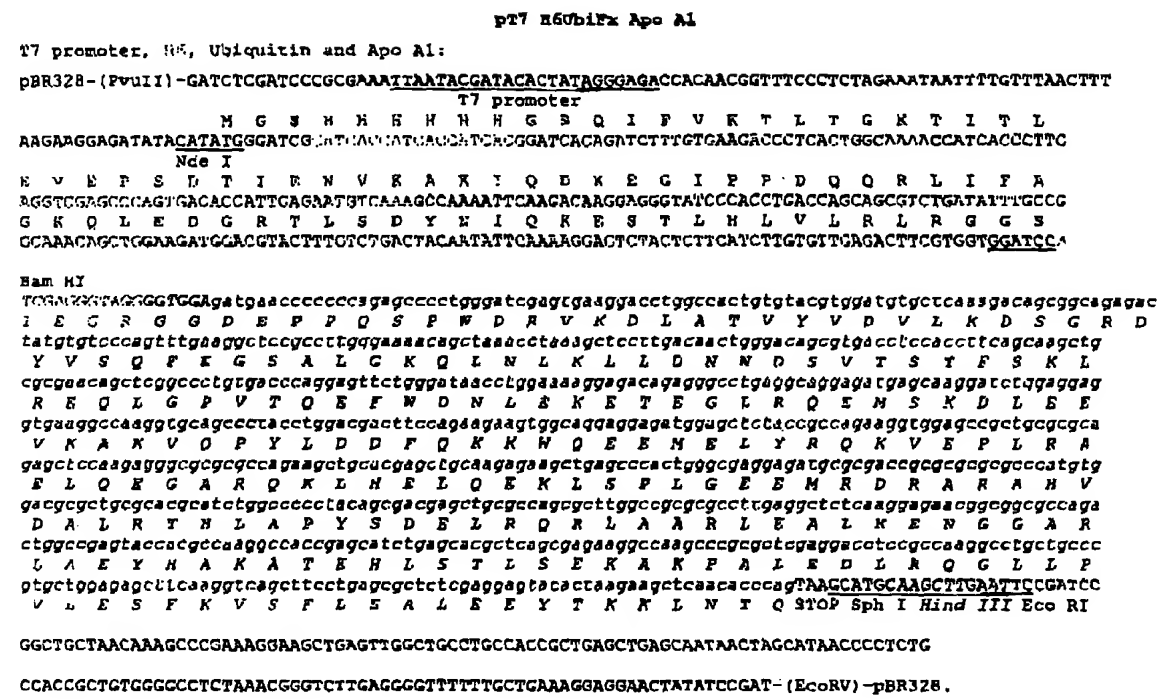

pT7 H6UbiFx Apo A1
T7 promoter, H6, Ubiquitin and Apo A1:
pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGATACACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT
T7 promoter
M G S H H H H H H G S Q I F V K T L T G K T I T L
AAGAAGGAGATATACATATGGGATCG
Nde I
E V E P S D T I E N V K A K I Q D K E G I P P D Q Q R L I F A
AGGTCGAGCCCAGTGACACCATTGAGAATGTCAAAGCCAAAATTCAAGACAAGGAGGGTATCCCACCTGACCAGCAGCGTCTGATATTTGCCG
G K Q L E D G R T L S D Y N I Q K E S T L H L V L R L R G G S
GCAAACAGCTGGAAGATGGACGTACTTTGTCTGACTACAATATTCAAAAGGAGTCTACTCTTCATCTTGTGTTGAGACTTCGTGGTGGATCCA
Bam HI
TCGAGCGGTAGCGGGTGGAgatgaaccccccgagccccctgggatcgagtgaaggacctggccactgtgtacgtggatgtgctcaaagacagcggcagagac
I E G R G G D E P P Q S P W D R V K D L A T V Y V D V L K D S G R D
tatgtgtcccagtttgaaggctccgccttgggaaaacagctaaacctaaagctccttgacaactgggacagcgtgacctccaccttcagcaagctg
Y V S Q F E G S A L G K Q L N L K L L D N W D S V T S T F S K L
cgcgaacagctcggccctgtgacccaggagttctgggataacctggaaaaggagacagagggcctgaggcaggagatgagcaaggatctggaggag
R E Q L G P V T Q E F W D N L E K E T E G L R Q E M S K D L E E
gtgaaggccaaggtgcagcccctacctggacgacttccagaagaagtggcaggaggagatggagctctaccgccagaaggtggagccgctgcgcgca
V K A K V Q P Y L D D F Q K K W Q E E M E L Y R Q K V E P L R A
gagctccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagcccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg
E L Q E G A R Q K L H E L Q E K L S P L G E E M R D R A R A H V
gacgcgctgcgcacgcatctggccccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaaggagaacggcggcgccaga
D A L R T H L A P Y S D E L R Q R L A A R L E A L K E N G G A R
ctggccgagtaccacgccaaggccaccgagcatctgagcacgctcagcgagaaggccaagcccgcgatcgaggacctccgccaaggcctgctgccc
L A E Y H A K A T E H L S T L S E K A K P A L E D L R Q G L L P
gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccagTAAGCATGCAAGCTTGAATTCCGATCC
V L E S F K V S F L S A L E E Y T K K L N T Q STOP Sph I Hind III Eco RI
GGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTCTG
CCACCGCTGTGGGCCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.

FIGURE 6

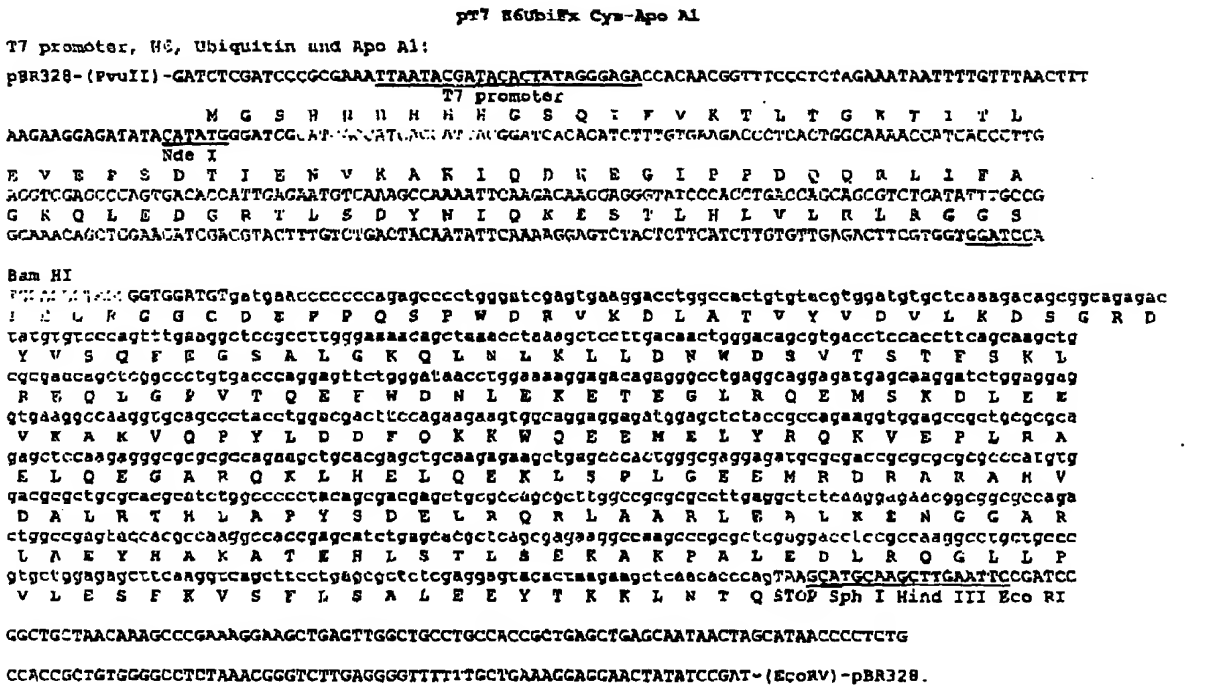

pT7 H6UbiFx Cys-Apo A1
T7 promoter, H6, Ubiquitin and Apo A1:
pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGATACACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT
T7 promoter
M G S H H H H H H G S Q I F V K T L T G K T I T L
AAGAAGGAGATATACATATGGGATCGCATCACATCACCATCACCATGGATCACACATCTTTGTGAAGACCCTCACTGGCAAAACCATCACCCTTG
Nde I
E V E P S D T I E N V K A K I Q D K E G I P P D Q Q R L I F A
AGGTCGAGCCCAGTGACACCATTGAGAATGTCAAAGCCAAAATTCAAGACAAGGAGGGTATCCCACCTGACCAGCAGCGTCTGATATTTGCCG
G K Q L E D G R T L S D Y N I Q K E S T L H L V L R L R G G S
GCAAACAGCTCGAACATCGACGTACTTTGTCTGACTACAATATTCAAAAGGAGTCTACTCTTCATCTTGTGTTGAGACTTCGTGGTGGATCCA
Bam HI
GGTGGATGTgatgaaccccccccagagcccctgggatcgagtgaaggacctggccactgtgtacgtggatgtgctcaaagacagcggcagagac
I E L R G G C D E F P Q S P W D R V K D L A T V Y V D V L K D S G R D
tatgtgtcccagtttgaaggctccgccttgggaaaacagctaaacctaaagctccttgacaactgggacagcgtgacctccaccttcagcaagctg
Y V S Q F E G S A L G K Q L N L K L L D N W D S V T S T F S K L
cgcgaacagctcggccctgtgacccaggagttctgggataacctggaaaaggagacagagggcctgaggcaggagatgagcaaggatctggaggag
R E Q L G P V T Q E F W D N L E K E T E G L R Q E M S K D L E E
gtgaaggccaaggtgcagccctacctggacgacttccagaagaagtggcaggaggagatggagctctaccgccagaaggtggagccgctgcgcgca
V K A K V Q P Y L D D F Q K K W Q E E M E L Y R Q K V E P L R A
gagctccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagcccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg
E L Q E G A R Q K L H E L Q E K L S P L G E E M R D R A R A H V
gacgcgctgcgcacgcatctggcccccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaagggaGAACggcggcgccaga
D A L R T H L A P Y S D E L R Q R L A A R L E A L K E N G G A R
ctggccgagtaccacgccaaggccaccgagcatctgagcacgctcagcgagaaggccaagcccgcgctcgaggacctccgccaaggcctgctgccc
L A E Y H A K A T E H L S T L S E R A K P A L E D L R Q G L L P
gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccagTAAGCATGCAAGCTTGAATTCCGATCC
V L E S F K V S F L S A L E E Y T K K L N T Q STOP Sph I Hind III Eco RI
GGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTTGG
CCACCGCTGTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.

FIGURE 7

pT7H6 Trip-A-Apo A1 - Amp^R.

T7 promoter, H6, FX$_a$-cleavage site and Trip-A insert sequence:

pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGATACACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGAT

T7 promoter

M G S H H H H H H G S I Q G R S P G T E P P T Q K P K K I V N A K K

ATACATATGGGATCGCATCACCATCACCATCACGGATCGATCCAAGGTAGATCTCCTGGTACCGAGCCACCAACCCAGAAGCCCAAGAAGATTGTAAATGCCAAGAAA

Bgl II Kpn I

D V V N T K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T V S L

GATGTTGTGAACACAAAGATGTTTGAGGAGCTCAAGAGCCGTCTGGACACCCTGGCCCAGGAGGTGGCCCTGCTGAAGGAGCAGCAGGCCCTGCAGACGGTCTCCCTG

Bam HI

AAGGGATCCgatgaacccccccagagcccctgggatcgagtgaaggacctggccactgtgtacgtggatgtgctcaaagacagcggcagagac

K G S *D E P P Q S P W D R V K D L A T V Y V D V L K D S G R D*

tatgtgtcccagtttgaaggctccgccttgggaaaacagctaaacctaaagctccttgacaactgggacagcgtgacctccaccttcagcaagctg

Y V S Q F E G S A L G K Q L N L K L L D N W D S V T S T F S K L

cgcgaacagctcggccctgtgacccaggagttctgggataacctggaaaaggagacagagggcctgaggcaggatgagcaaggatctggaggag

R E Q L G P V T Q E F W D N L E K E T E G L R Q E M S K D L E E

gtgaaggccaaggtgcagccctacctggacgacttccagaagaagtggcaggaggagatggagctctaccgccagaaggtggagccgctgcgcgca

V K A K V Q P Y L D D F Q K K W Q E E M E L Y R Q K V E P L R A

gagctccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagcccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg

E L Q E G A R Q K L H E L Q E K L S P L G E E M R D R A R A H V

gacgcgctgcgcacgcatctggcccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaaggagaacggcggcgccaga

D A L R T H L A P Y S D E L R Q R L A A R L E A L K E N G G A R

ctggccgagtaccacgccaaggccaccgagcatctgagcacgctcagcgagaaggccaagcccgcgctcgaggacctccgccaaggcctgctgccc

L A E Y H A K A T E H L S T L S E K A K P A L E D L R Q G L L P

*gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccag*TAATAAGCTTGAATTCCGATCCGGCTGCTAA

V L E S F K V S F L S A L E E Y T K K L N T Q STOP Hind III Eco RI

CAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTTGTGCCACCGCTGTGGGGCCTCTAAACGGGTCTTGAGGGG

TTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.

FIGURE 8

pT7H6 Trip-A-Apo A1-del43 - Amp^R.

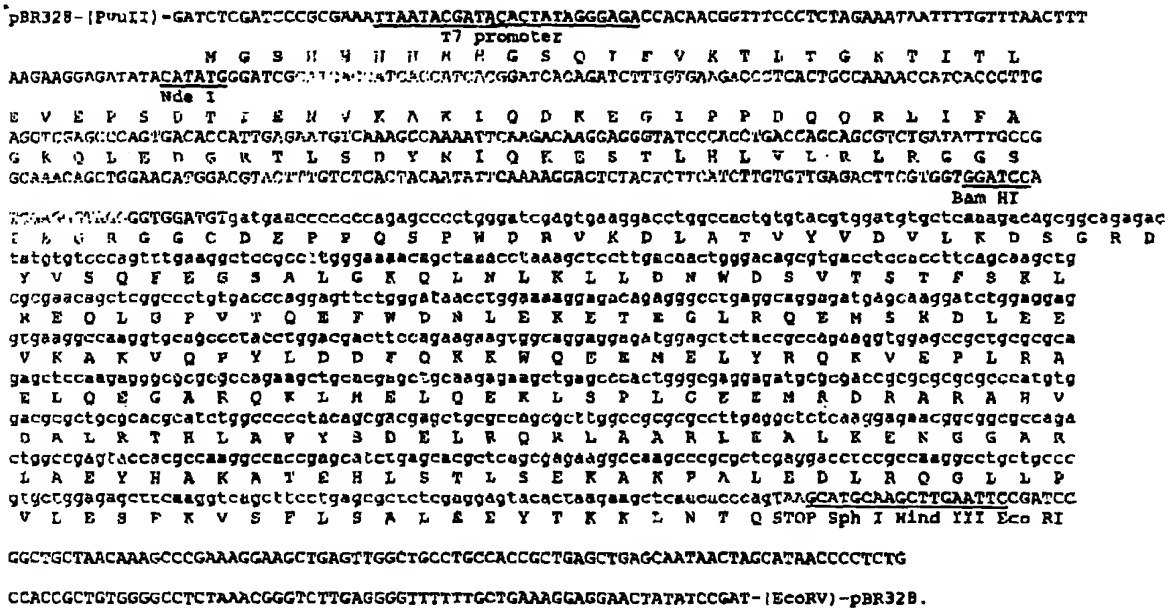
T7 promoter, H6, FX$_a$-cleavage site and Trip-A insert sequence:

```
pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGATACACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGAT
                                T7 promoter
      M  G  S  H  H  H  H  H  H  G  S  I  E  G  R  S  P  G  T  E  P  P  T  Q  K  P  K  K  I  V  N  A  K  K
ATACATATGGGATCGCATCACCATCACCATCACGGATCGATCCAGGGTAGATCTCCTGGTACCGAGCCACCAACCCAGAAGCCCAAGAAGATTGTAAATGCCAAGAAA
                                                        Bgl II   Kpn I
 D  V  V  N  T  K  M  F  E  E  L  K  S  R  L  D  T  L  A  Q  E  V  A  L  L  K  E  Q  Q  A  L  Q  T  V  S  L
GATGTTGTGAACACAAAGATGTTTGAGGAGCTCAAGAGCCGTCTGGACACCCTGGCCCAGGAGGTGGCCCTGCTGAAGGAGCAGCAGGCCCTGCAGACGGTCTCCCTG
  Bam HI
AAGGGATCCctaaagctccttgacaactgggacagcgtgacctccaccttcagcaagctg
 K  G  S  L  K  L  L  D  N  W  D  S  V  T  S  T  F  S  K  L

cgcgaacagctcggccctgtgacccaggagttctgggataacctggaaaaggagacagagggcctgaggcaggagatgagcaaggatctggaggag
 R  E  Q  L  G  P  V  T  Q  E  F  W  D  N  L  E  K  E  T  E  G  L  R  Q  E  M  S  K  D  L  E  E
gtgaaggccaaggtgcagccctacctggacgacttccagaagaagtggcaggaggagatggagctctaccgccagaaggtggagccgctgcgcgca
 V  K  A  K  V  Q  P  Y  L  D  D  F  Q  K  K  W  Q  E  E  M  E  L  Y  R  Q  K  V  E  P  L  R  A
gagctccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagcccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg
 E  L  Q  E  G  A  R  Q  K  L  H  E  L  Q  E  K  L  S  P  L  G  E  E  M  R  D  R  A  R  A  H  V
gacgcgctgcgcacgcatctggccccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaaggagaacggcggcgccaga
 D  A  L  R  T  H  L  A  P  Y  S  D  E  L  R  Q  R  L  A  A  R  L  E  A  L  K  E  N  G  G  A  R
ctggccgagtaccacgccaaggccaccgagcatctgagcacgctcagcgagaaggccaagcccgcgctcgaggacctccgccaaggcctgctgccc
 L  A  E  Y  H  A  K  A  T  E  H  L  S  T  L  S  E  K  A  K  P  A  L  E  D  L  R  Q  G  L  L  P
gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccagTAATAAGCTTGAATTCCGATCCGGCTGCTAA
 V  L  E  S  F  K  V  S  F  L  S  A  L  E  E  Y  T  K  K  L  N  T  Q STOP Hind III Eco RI

CAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTCTGCCACCGCTGTGGGGCCTCTAAACGGGTCTTGAGGGG
TTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.
```

FIGURE 9

pT7 H6UbiFx Cys-Apo A1

T7 promoter, H6, Ubiquitin and Apo A1:



SEQUENCE LISTING

<110> ProteoPharma ApS

<120> Apolipoprotein

<130> Apolipoprotein

<140>
<141>

<160> 4

<170> PatentIn Ver. 2.1

<210> 1
<211> 243
<212> PRT
<213> Homo sapiens

<400> 1

```
Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala Thr
 1               5                  10                  15

Val Tyr Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln
            20                  25                  30

Phe Glu Gly Ser Ala Leu Gly Lys Gln Leu Asn Leu Lys Leu Leu Asp
        35                  40                  45

Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu
    50                  55                  60

Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu
65                  70                  75                  80

Gly Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala Lys
                85                  90                  95

Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu Met
            100                 105                 110

Glu Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln Glu
        115                 120                 125

Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro Leu
    130                 135                 140
```

```
Gly Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu Arg
145                 150                 155                 160

Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala
                165                 170                 175

Arg Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr
            180                 185                 190

His Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala Lys
        195                 200                 205

Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu Ser
    210                 215                 220

Phe Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu
225                 230                 235                 240

Asn Thr Gln
```

<210> 2
<211> 244
<212> PRT
<213> Homo sapiens

<400> 2

```
Cys Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala
  1               5                  10                  15

Thr Val Tyr Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr Val Ser
            20                  25                  30

Gln Phe Glu Gly Ser Ala Leu Gly Lys Gln Leu Asn Leu Lys Leu Leu
        35                  40                  45

Asp Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg Glu Gln
    50                  55                  60

Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr
65                  70                  75                  80

Glu Gly Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala
                85                  90                  95

Lys Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu
            100                 105                 110
```

Met Glu Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln
115 120 125

Glu Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro
130 135 140

Leu Gly Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu
145 150 155 160

Arg Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala
165 170 175

Ala Arg Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu
180 185 190

Tyr His Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala
195 200 205

Lys Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu
210 215 220

Ser Phe Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys
225 230 235 240

Leu Asn Thr Gln

<210> 3
<211> 301
<212> PRT
<213> Homo sapiens

<400> 3
Ser Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn
1 5 10 15

Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser
20 25 30

Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln
35 40 45

Ala Leu Gln Thr Val Ser Leu Lys Gly Ser Asp Glu Pro Pro Gln Ser
50 55 60

Pro Trp Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu

65 70 75 80

Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu
85 90 95

Gly Lys Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr
100 105 110

Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu
115 120 125

Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met
130 135 140

Ser Lys Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp
145 150 155 160

Asp Phe Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys
165 170 175

Val Glu Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu
180 185 190

His Glu Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp
195 200 205

Arg Ala Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr
210 215 220

Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys
225 230 235 240

Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu
245 250 255

His Leu Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu
260 265 270

Arg Gln Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Phe Leu
275 280 285

Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu Asn Thr Gln
290 295 300

<210> 4
<211> 258
<212> PRT

<213> Homo sapiens

<400> 4

Ser Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn
1 5 10 15

Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser
20 25 30

Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln
35 40 45

Ala Leu Gln Thr Val Ser Leu Lys Gly Ser Leu Lys Leu Leu Asp Asn
50 55 60

Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu Gly
65 70 75 80

Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu Gly
85 90 95

Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala Lys Val
100 105 110

Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu Met Glu
115 120 125

Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln Glu Gly
130 135 140

Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro Leu Gly
145 150 155 160

Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu Arg Thr
165 170 175

His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg
180 185 190

Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His
195 200 205

Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala Lys Pro
210 215 220

Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu Ser Phe
225 230 235 240

Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu Asn
245 250 255

Thr Gln

Kongeriget Danmark

Patent application No.: PA 2000 01682

Date of filing: 10 November 2000

Applicant: ProteoPharma ApS
c/o Østjysk Innovation
Forskerparken, Gustav Wied Vej 10
DK-8000 Århus C

This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

- The specification, claims, figures and sequence listing as filed with the application on the filing date indicated above.

Patent- og Varemærkestyrelsen • Kongeriget Danmark •

Patent- og Varemærkestyrelsen
Erhvervsministeriet

Taastrup, 28 November 2001

Karin Schlichting
Head Clerk

Apolipoprotein analogues

The invention relates to an apolipoprotein construct, a nucleic acid sequence encoding the apolipoprotein construct, a vector comprising the nucleic acid sequence, a method for producing the apolipoprotein construct, a pharmaceutical composition comprising the apolipoprotein construct, and use of the apolipoprotein construct for the preparation of a pharmaceutical composition.

Prior art

In the following, the term Apo A or apolipoprotein A will be used to designate any of the three apolipoproteins, Apolipoprotein A I, Apolipoprotein A II, or Apolipoprotein A III.

Atherosclerosis is the most frequent cause of death in the industrialised countries of the World. One of the pathogenic factors causing atherosclerosis is the deposition of cholesterol in the blood vessels, which ultimately leads to clogging of the vessels.

Apolipoprotein A-1 (apo-A-1) is the main component of plasma HDL (high density lipoprotein), which is negatively correlated to the presence of arteriosclerosis. There is strong experimental evidence that this effect is caused by so-called reverse cholesterol transport from peripheral tissues to the liver. There is also experimental evidence that this reverse cholesterol transport can be stimulated in mammals by injection of apo-A-1.

Apolipoprotein A is to a large extent removed from plasma by filtration in the kidneys without being broken down first. The short plasma half-life of apolipoprotein A is a constraint against using the protein in the treatment of atherosclerosis.

US 5,876,968 (SIRTORI ET AL.) concerns substantially pure dimers of a variant of apo-A-1 called apolipoprotein A-1-Milano. Medicaments containing the dimer can be used for preventing thrombosis or they can be used as a prodrug for the monomer. A specific feature of this particular variant of apo-A-I is its ability to form covalent dimers with itself. The authors found that the purified dimer Apo A-I-M/Apo A-I-M has a prolonged plasma half-life compared to the monomer Apo A-I-M.

US 5,643,757 (SHA-IL ET AL.) discloses a method for the production of pure, stable, mature and biologically active human apolipoprotein A-I in high yield.

US 5,990,081 (AGELAND ET AL.) discloses a method for treatment of arteroscierosis or cardiovascular diseases by administering a therapeutically effective amount of apoliproprotein A or apolipoprotein E.

WO 96/37608 (RHONE-POULENC ROHRER ET AL.) describes humane homologous dimers of apolipoprotein A-I variants comprising cystein in position 151. The presence of the cystein residue in the amino acid sequence allows the formation of dimers via disulphide bridges between the monomers. The reference furthermore discloses the corresponding nucleic acid sequences and vectors comprising these as well as pharmaceutical compositions comprising the variants and the use of these in gene therapy.

Summary

In a first aspect the invention relates to an apolipoprotein construct having the general formula

- apo-X,
- where apo is an apolipoprotein component selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, an analogue or a variant thereof,
- and X is a heterologous moiety comprising at least one compound selected from the group consisting of an amino acid, a non-apolipoprotein peptide, an oligomerising module, a carbohydrate, a nucleic acid sequence, a non-apolipoprotein protein, and an apolipoprotein selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, an analogue or a variant thereof,
- with the proviso that when the construct consists of exactly two identical, native apolipoproteins these are linked serially.

Throughout the invention the apolipoprotein component or part of the construct is referred to as apo or apolipoprotein. In the following and in the claims, the

heterologous moiety is referred to as component X of the construct. The apolipoprotein or analogue or variant thereof is linked covalently to the heterologous moiety.

The component X of the construct may be looked broadly upon as a heterologous moiety. In this context a heterologous moiety is any kind of moiety not being linked to apolipoprotein or analogue or variant or functional equivalent thereof under native conditions. The heterologous moiety may thus be a peptide or a protein or part of a peptide or protein from the same or from another species, or even a single amino acid. It may be a synthetic peptide. It may be of carbohydrate nature or of other polymeric and biocompatible nature such as polyols, nucleic acids sequences.

Functional equivalence to native apolipoprotein A-I, A-II or A-IV may conveniently be measured using a lipid binding assay. The ability of the construct to elicit substantially the same physiological response in a mammal may conveniently be measured by measurement of the ability to perform reverse cholesterol transport in a test organism such as rabbits.

The construct comprising apolipoprotein and a heterologous moiety is capable of performing reverse cholesterol transport as well as or even better than native apolipoproteins, despite the modification caused by the addition of a heterologous moiety. The plasma half-life of the construct is preferably increased compared to that of the wild-type apolipoprotein. Preferably the plasma half-life is at least such as doubled or tripled, or at least quadrupled, or at least 10 doubled. Similarly, the binding affinity such as the lipid binding affinity, and/or the cholesterol binding affinity of the construct is preferably increased as compared to wild-type apolipoprotein. Preferably, the lipid binding affinity is increased by at least 5 %, such as at least 10 %, for example at least 15%, such as at least 20%, for example at least 25%, such as at least 30%, for example at least 40% such as at least 50%, for example at least 75%, such as at least 100%, such as at least 150%, for example at least 200%, such as at least 300%.

An increased plasma half-time and/or increased lipid binding affinity have profound implications for the use of the apolipoprotein constructs in the treatment of arteriosclerosis. It is therefore expected that the clinical effect of the apolipoprotein

constructs according to the invention is sup rior to the effect of wild-type apolipoproteins.

The invention also encompasses analogues or variants of the wild-type apoliproteins capable of eliciting substantially the same physiological response in a mammal.

According to a second aspect of the invention, there is provided a nucleotide sequence encoding an apolipoprotein construct as defined above. Preferably the nucleotide sequence is operably linked to a regulatory sequence for expression of the protein construct.

According to further aspects of the invention, there is provided a vector comprising the nucleotide sequence encoding the apolipoprotein construct and a transformed host cell comprising the nucleotide sequence as defined above.

The apolipoprotein construct according to the invention may be produced by different methods.

According to a first method a transformed host cell is cultured under conditions promoting the expression of a protein construct according to the invention encoded by DNA inserted into a construct, obtaining and recovering the protein construct and optionally further processing the protein construct.

This method is the preferred method when the whole construct is of polypeptide nature and thus can be encoded by one corresponding nucleic acid sequence.

According to a second method the apolipoprotein construct can be manufactured by chemically synthesising the heterologous moiety and subsequently linking it to the apolipoprotein or analogue obtaining an apolipoprotein construct, which is isolated and optionally processed further. This method is the preferred method, when the heterologous moiety is of non-peptide nature. However there may also be conditions under which it is preferred to synthesise the heterologous moiety chemically, when it is of polypeptide nature. Such conditions may be that the heterologous moiety is rather short such as below 20 amino acids.

According to a third method the apolipoprotein construct can be manufactured by culturing a transformed host cell under conditions promoting the expression of an apolipoprotein or an apolipoprotein analogue encoded by a nucleic acid fragment and subsequently covalently linking the apolipoprotein or apolipoprotein analogue to a heterologous moiety obtaining an apolipoprotein construct, isolating the resulting apolipoprotein construct and optionally further processing the construct.

Finally, the apolipoprotein construct may be produced by culturing a transformed host cell under conditions promoting the expression of a protein encoded by a nucleic acid fragment encoding an oligomerising module and subsequently linking said module to at least one apolipoprotein obtaining an apolipoprotein construct.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising the apolipoprotein construct as described above. Preferably the pharmaceutical composition is capable of being administered parenterally such as through injection.

The invention also encompasses the use of an apolipoprotein construct as defined above for the preparation of a pharmaceutical composition. The pharmaceutical composition may further comprise pharmaceutical acceptable excipients, adjuvants, additives, such as lipids, phospholipids, cholesterol, or triglycerides.

The pharmaceutical composition may be administered intravenously, intraarterially, intramuscularly, transdermally, pulmonary, subcutaneously, intradermally, intratechally, through the buccal-, anal-, vaginal-, conjunctival-, or intranasal tissue, or by inoculation into tissue, such as tumour tissue, or by an implant, or orally.

Preferably the pharmaceutical composition is used for the treatment and/or prevention of aterosclerose, angina pectoris, claudicatio, or in the treatment of neutralising endotoxins. The apolipoprotein construct as defined above may also be used for gene therapy, wherein the DNA sequence encoding the apolipoprotein construct is used for transfection or infection of at least one cell population.

Detailed description of the invention

In the following the invention will be described in detail with reference to the following figures.

Figure 1 shows the amino acid sequence (in one letter code) of human apolipoprotein A-I.

Figure 2 shows aligned amino acid sequences (in one letter code) for human, macaque, mouse, baboon, pig, and rat apolipoprotein A-IV.

Figure 3: Amino acid sequence of the amino terminal region of tetranectin. Amino acid sequence (in one letter code) from E1 to L51 of tetranectin. Exon 1 comprises residues E1 to D16 and exon 2 residues V17 to V49, respectively. The alpha helix extends beyond L51 to K52 which is the C-terminal amino acid residue in the alpha helix.

Figure 4 shows an alignment of the amino acid sequences of the trimerising structural element of the tetranectin protein family. Amino acid sequences (one letter code) corresponding to residue V17 to K52 comprising exon 2 and the first three residues of exon 3 of human tetranectin; murine tetranectin (Sørensen et al., Gene, 152: 243 -245, 1995); tetranectin homologous protein isolated from reefshark cartilage (Neame and Boynton, 1992,1996); and tetranectin homologous protein isolated from bovine cartilage (Neame and Boynton, database accession number PATCHX:u22298). Residues at a and d positions in the heptad repeats are listed in boldface. The listed consensus sequence of the tetranectin protein family trimerising structural element comprise the residues present at a and d positions in the heptad repeats shown in the figure in addition to the other conserved residues of the region. "hy" denotes an aliphatic hydrophobic residue.

Figure 5 shows the pT7 H6UbiFx Apo A-I plasmid and its corresponding amino acid sequences. The expressed and purified polypeptide (SEQ ID NO 1) consists of amino acids no 25-267 from human Apo A-I.

Figure 6 shows the pT7 H6UbiFx Cys-Apo A-I plasmid and its corresponding amino acid sequences for. The expressed polypeptide (SEQ ID NO 2) consists of a N-terminal cystein residue and the amino acids no 25-267 from human Apo A-I.

Figure 7 shows the pT7H6 Trip-A-Apo A-I - Amp^R plasmid and its corresponding amino acid sequence. The expressed and purified polypeptide (SEQ ID NO 3) consists of the TTSE, a linking sequence, and amino acids no 25-267 from human Apo A-I.

Figure 8 shows the pT7H6 Trip-A-Apo A-I-del 43 - Amp^R plasmid and its corresponding amino acid sequence. The expressed and purified polypeptide (SEQ ID NO 4) consists of the TTSE, a linking sequence, and amino acids no 68-267 from human Apo A-I.

Figure 9 shows the pT7H6FXCysApoAI plasmid and its corresponding amino acid sequence. The expressed and purified polypeptide (SEQ ID NO 2) consists of a N-terminal cystein residue and the amino acids no 25-267 from human Apo A-I.

Detailed description of the invention

The functionality of the constructs according to the invention and of the apo components of the constructs can be measured by a lipid binding assay such as by the DPMC assay described below. Furthermore, the in vivo effect on reverse cholesterol transport may be measured by administration to test animals such as rabbits fed on a cholesterol rich diet such as the method disclosed in Miyazaki et al (Arteriosclerosis, Thrombosis, and Vascular Biology, 1995; 15:1882-1888).

Kinetics of association of the protein construct with dimyristoyl phosphatidylcholine (DMPC)

The ability of the constructs according to the invention to bind to a lipid can conveniently be measured using a well known assay such as the association to dimyristoyl phosphatidylcholine (DPMC).

The assay was conducted as described in (Pownall et al, Biochemistry, 1978, 17: 83-89). Dried DPMC was suspended in 100 mM NaCl, 50 mM Tris-HCl pH 8.0 above its transition temperature at a concentration of 1 mg/ml. The protein sample and the DMPC suspension was both preincubated at room temperature for 10

minutes, and then mixed at a ratio of DMPC:protein of 50:1. The reduction in turbidity of the mixture, reflecting increasing lipid-protein association, was followed by measuring the absorbance of the mixture at 325 nm. The turbidity clearance curves were fitted to a double exponential equation, and $t_{1/2}$ were determined.

The apolipoprotein or analogue

In the following the term "apo" is used to designate any protein comprising apolipoprotein A-I, apolipoprotein A-II or apolipoprotein A-IV, any variant or analogue thereof possessing the same lipid binding function.

Preferred apolipoprotein A-I analogues include those disclosed in Figures 5, 6, 7 and 8 and listed as SEQ ID NO 1, 2, 3, and 4.

Known variants of the sequences in Figure 1 include the following variants, indicating the position of the variation with respect to the sequence in Figure 1, the variation, and where appropriate the name of the known variant.

27 P -> H (IN MUNSTER-3C).
27 P -> R.
28 P -> R (IN MUNSTER-3B).
34 R -> L (IN BALTIMORE).
50 G -> R (IN IOWA).
84 L -> R (IN AUTOSOMAL DOMINANT AMYLOIDOSIS).
113 D -> E.
119 A -> D (IN HITA).
127 D -> N (IN MUNSTER-3A).
131 MISSING (IN MARBURG/MUNSTER-2).
131 K -> M.
132 W -> R (IN TSUSHIMA).
134 E -> K (IN FUKUOKA).
160 E -> K (IN NORWAY).
163 E -> G.
167 P -> R (IN GIESSEN).
168 L -> R (IN ZARAGOZA).
171 E -> V.

189 P -> R.
197 R -> C (IN MILANO).
222 E -> K (IN MUNSTER-4).

According to the invention the term "apolipoprotein" is meant to include functional equivalents of at least one sequence in Figure 1 and 2, or a fragment of at least one sequence in Figure 1 and 2, comprising a predetermined amino acid sequence. A "fragment" is defined as:

i) fragments comprising an amino acid sequence capable of being recognised by an antibody also capable of recognising the predetermined amino acid sequences in Figure 1 or 2, and/or

ii) fragments comprising an amino acid sequence capable of binding to a lipid (DPMC assay), which is also capable of binding the predetermined amino acid sequences in Figure 1 or 2.

According to the present invention a functional equivalent of an apolipoprotein or fragments thereof may be obtained by addition, substitution or deletion of at least one amino acid. When the amino acid sequence comprises a substitution of one amino acid for another, such a substitution may be a conservative amino acid substitution. Fragments of the sequences in Figure 1 and 2 may comprise more than one such substitution, such as e.g. two conservative amino acid substitutions, for example three or four conservative amino acid substitutions, such as five or six conservative amino acid substitutions, for example seven or eight conservative amino acid substitutions, such as from 10 to 15 conservative amino acid substitutions, for example from 15 to 25 conservative amino acid substitution, such as from 25 to 75 conservative amino acid substitutions, for example from 75 to 125 conservative amino acid substitutions, such as from 125 to 175 conservative amino acid substitutions. Substitutions can be made within any one or more groups of predetermined amino acids.

Examples of fragments comprising one or more conservative amino acid substitutions including one or more conservative amino acid substitutions within the same group of predetermined amino acids, or a plurality of conservative amino acid

substitutions, wherein each conservative substitution is generated by substitution within a different group of predetermined amino acids.

Accordingly, a variant of the sequences in Figure 1 or 2, or fragments thereof according to the invention may comprise, within the same variant of the sequences in Figure 1 or 2, or fragments thereof or among different variant of the sequences in Figure 1 or 2, or fragments thereof, at least one substitution, such as a plurality of substitutions introduced independently of one another. Variants of the sequences in Figure 1 or 2, or fragments thereof may thus comprise conservative substitutions independently of one another, wherein at least one glycine (Gly) of said variants of the sequences in Figure 1 or 2, or fragments thereof of the sequences in Figure 1 or 2 is substituted with an amino acid selected from the group of amino acids consisting of Ala, Val, Leu, and Ile, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said alanines (Ala) of said variant of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Val, Leu, and Ile, and independently thereof, variant of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one valine (Val) of said variant of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Leu, and Ile, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said leucines (Leu) of said variant of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Val, and Ile, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one isoleucine (Ile) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Val and Leu, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof wherein at least one of said aspartic acids (Asp) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Glu, Asn, and Gln, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said phenylalanines (Phe) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of

amino acids consisting of Tyr, Trp, His, Pro, and preferably selected from the group of amino acids consisting of Tyr and Trp, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said tyrosines (Tyr) of said variants of the sequences in Figure 1 or 2, or fragments thereof of the sequences in Figure 1 or 2 is substituted with an amino acid selected from the group of amino acids consisting of Phe, Trp, His, Pro, preferably an amino acid selected from the group of amino acids consisting of Phe and Trp, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said arginines (Arg) of said fragment of the sequences in Figure 1 or 2 is substituted with an amino acid selected from the group of amino acids consisting of Lys and His, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one lysine (Lys) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Arg and His, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said aspargines (Asn) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Gln, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one glutamine (Gln) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Asn, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one proline (Pro) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Phe, Tyr, Trp, and His, and independently thereof, variants of the sequences in Figure 1 or 2, or fragments thereof, wherein at least one of said cysteines (Cys) of said variants of the sequences in Figure 1 or 2, or fragments thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, and Tyr.

It is clear from the above outline that the same variant or fragment thereof may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

The addition or deletion of an amino acid may be an addition or deletion of from 2 to 10 amino acids, such as from 10 to 20 amino acids, for example from 20 to 30 amino acids, such as from 40 to 50 amino acids. However, additions or deletions of more than 50 amino acids, such as additions from 10 to 200 amino acids, are also comprised within the present invention. More specifically, 43 N-terminal amino acids may be removed from the sequence in Figure 1 without substantially altering the lipid binding effect of the protein. Such a deletion variant is included in SEQ ID NO 4 as the apolipoprotein part of the construct.

It will thus be understood that the invention concerns apolipoproteins comprising at least one fragment of the sequences in Figure 1 or 2 capable of binding lipids such as DPMC, including any variants and functional equivalents of such at least one fragment.

The apolipoprotein according to the present invention, including any functional equivalents and fragments thereof, may in one embodiment comprise less than 243 amino acid residues, such as less than 240 amino acid residues, for example less than 225 amino acid residues, such as less than 200 amino acid residues, for example less than 180 amino acid residues, such as less than 160 amino acid residues, for example less than 150 amino acid residues, such as less than 140 amino acid residues, for example less than 130 amino acid residues, such as less than 120 amino acid residues, for example less than 110 amino acid residues, such as less than 100 amino acid residues, for example less than 90 amino acid residues, such as less than 85 amino acid residues, for example less than 80 amino acid residues, such as less than 75 amino acid residues, for example less than 70 amino acid residues, such as less than 65 amino acid residues, for example less than 60 amino acid residues, such as less than 55 amino acid residues, for example less than 50 amino acid residues.

Fragments

A fragment comprising the lipid binding region of the native sequences in Figure 1 or 2 is particularly preferred. However, the invention is not limited to fragments comprising the lipid binding region. Deletions of such fragments generating functionally equivalent fragments of the sequences in Figure 1 or 2 comprising less

than the lipid binding region are also comprised in the present invention. Functionally equivalent the sequences in Figure 1 or 2 peptides, and fragments thereof according to the present invention, may comprise less or more amino acid residues than the lipid binding region. Preferably, the fragment comprises at least the amino acids 100-186 of apo-A-I or a variant or a functional equivalent thereof. It has been determined that this central domain and the α-helices within the domain are directly involved in interactions with phospholipids. Therefore, it is highly likely that this region plays an important role in the functional properties of apo-A-I.

"Functional equivalency" as used in the present invention is according to one preferred embodiment established by means of reference to the corresponding functionality of a predetermined fragment of the sequences in Figure 1 or 2.

Functional equivalents of variants of the sequences in Figure 1 or 2 will be understood to exhibit amino acid sequences gradually differing from the preferred predetermined sequence, as the number and scope of insertions, deletions and substitutions including conservative substitutions increases. This difference is measured as a reduction in homology between the preferred predetermined sequence and the fragment or functional equivalent.

All fragments or functional equivalents of apolipoprotein are included within the scope of this invention, regardless of the degree of homology that they show to a preferred predetermined sequence of apolipoprotein. The reason for this is that some regions of the sequences in Figure 1 or 2 are most likely readily mutatable, or capable of being completely deleted, without any significant effect on the binding activity of the resulting fragment.

A functional variant obtained by substitution may well exhibit some form or degree of native activity of the sequences in Figure 1 or 2, and yet be less homologous, if residues containing functionally similar amino acid side chains are substituted. Functionally similar in this respect refers to dominant characteristics of the side chains such as hydrophobic, basic, neutral or acidic, or the presence or absence of steric bulk. Accordingly, in one embodiment of the invention, the degree of identity between I) a given the sequences in Figure 1 or 2 fragment capable of effect and II) a preferred predetermined fragment, is not a principal measure of the fragment as a

variant or functional equivalent of a preferred predetermined the sequences in Figure 1 or 2 fragment according to the present invention.

The homology between amino acid sequences may be calculated using well known algorithms such as BLOSUM 30, BLOSUM 40, BLOSUM 45, BLOSUM 50, BLOSUM 55, BLOSUM 60, BLOSUM 62, BLOSUM 65, BLOSUM 70, BLOSUM 75, BLOSUM 80, BLOSUM 85, or BLOSUM 90. Preferably the algorithm BLOSUM 30 is used.

Fragments sharing at least some homology with the sequences in Figure 1 or 2 fragment are to be considered as falling within the scope of the present invention when they are at least about 40 percent homologous with the apolipoprotein or fragment thereof, such as at least about 50 percent homologous, for example at least about 60 percent homologous, such as at least about 70 percent homologous, for example at least about 75 percent homologous, such as at least about 80 percent homologous, for example at least about 85 percent homologous, such as at least about 90 percent homologous, for example at least 92 percent homologous, such as at least 94 percent homologous, for example at least 95 percent homologous, such as at least 96 percent homologous, for example at least 97 percent homologous, such as at least 98 percent homologous, for example at least 99 percent homologous with the sequences in Figure 1 or 2 fragment. According to one embodiment of the invention the homology percentages refer to identity percentages.

Additional factors that may be taken into consideration when determining functional equivalence according to the meaning used herein are i) the ability of antisera against one of the sequences in Figure 1 or 2 to detect fragments of the sequences in Figure 1 or 2 according to the present invention, or ii) the ability of the functionally equivalent fragment to compete with the sequences in Figure 1 or 2 in a lipid binding assay.

Conservative substitutions may be introduced in any position of a preferred predetermined apolipoprotein or fragment thereof. It may however also be desirable to introduce non-conservative substitutions, particularly, but not limited to, a non-conservative substitution in any one or more positions.

A non-conservative substitution leading to the formation of a functionally equivalent fragment of the sequences in Figure 1 or 2 would for example i) differ substantially in polarity, for example a residue with a non-polar side chain (Ala, Leu, Pro, Trp, Val, Ile, Leu, Phe or Met) substituted for a residue with a polar side chain such as Gly, Ser, Thr, Cys, Tyr, Asn, or Gln or a charged amino acid such as Asp, Glu, Arg, or Lys, or substituting a charged or a polar residue for a non-polar one; and/or ii) differ substantially in its effect on polypeptide backbone orientation such as substitution of or for Pro or Gly by another residue; and/or iii) differ substantially in electric charge, for example substitution of a negatively charged residue such as Glu or Asp for a positively charged residue such as Lys, His or Arg (and vice versa); and/or iv) differ substantially in steric bulk, for example substitution of a bulky residue such as His, Trp, Phe or Tyr for one having a minor side chain, e.g. Ala, Gly or Ser (and vice versa).

Substitution of amino acids may in one embodiment be made based upon their hydrophobicity and hydrophilicity values and the relative similarity of the amino acid side-chain substituents, including charge, size, and the like. Exemplary amino acid substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition to the variants described herein, sterically similar variants may be formulated to mimic the key portions of the variant structure and that such compounds may also be used in the same manner as the variants of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

The component X

Preferably, the component X of the protein construct according to the invention is essentially non-immunogenic. For instance the component X may be an amino acid, a carbohydrate, a nucleic acid sequence, an inert protein or polypeptide, which has

substantially no physiological effect and especially no immunological effect on mammals.

According to one embodiment the component X consists of just one amino acid, which amino acid preferably is a cystein residue, which may be placed N-terminally, C-terminally or internally in the apolipoprotein component.

However, the component X may also comprise a peptide having more than 1 amino acids such as more than 2 amino acids, for example more than 5 amino acids, such as more than 10 amino acids, for example more than 15 amino acids, such as more than 20 amino acids, such as more than 30 amino acids, for example more than 40 amino acids, such as more than 50 amino acids, for example more than 75 amino acids, such as more than 100 amino acids, for example more than 200 amino acids, such as more than 300 amino acids, for example more than 400 amino acids, such as more than 500 amino acids, for example more than 600 amino acids, such as more than 700 amino acids, for example more than 800 amino acids, such as more than 900 amino acids, for example more than 1000, 1250, 1500, 2000, or 2500 amino acids.

One protein could be a plasma protein such as albumin or another non-immunogenic peptide or protein.

According to an especially preferred embodiment of the invention, the component X comprises an apolipoprotein component selected from the group consisting of apolipoprotein A-I, A-II, AIV, an analogue, functional variant or fragment thereof. The two apolipoprotein components may be linked linearly or they may be linked via a non-native cysein bridge. Preferably they are linked linearly.

Higher oligomers as well as dimers of the apolipoprotein component comprising at least one non-native cystein residue may be manufactured and linked through cystein bridges under appropriate conditions. Oligomers linked by disulphide bridges may be linked serially (apo-S-S-apo, or apo-S-S-apo-S-S-apo or higher oligomers) or the disulphide bridge may be formed non-terminally by inserting a cystein residue at a non-terminal position in each of the two proteins to be linked and linking the two proteins through a cystin bridge. By inserting two, three or more cystein residues

into one or more of the proteins trimers, tetramers and higher multimers may be formed.

The protein construct according to the invention may also comprise two, three or more apolipoproteins or analogues thereof being serially and covalently linked to one another. This may be achieved by linking the C-terminal of a first apolipoprotein to the N-terminus of the next apolipoprotein and so forth. The proteins may be so linked after transcription and translation or the nucleotide sequence may simply comprise two, three or more sequences coding for the apolipoprotein construct in question as well as optional linker peptides between the apolipoproteins.

Thereby, the need for a heterologous moiety to perform the linkage is avoided. It is expected that in the constructs having two, three or more apo units essentially all the apo units will participate in lipid binding thereby contributing to the functionality of the construct. Therefore it is expected that these multi-apo constructs may have an increased lipid binding ability compared to native apo. An additional advantage of these constructs compared to native apo, is that they have an increased plasma half-life compared to native apo.

Such constructs comprising more than one apolipoprotein component may comprise a combination selected from the following group:

Dimers:
A-I A-I; A-II AII; A-IV AIV; A-I A-II; A-I A-IV; A-II A-IV.

Trimers:
A-I A-II A-IV; A-I A-I A-II; A-I A-I A-I; A-I A-I A-IV; A-II A-II A-I; A-II A-II A-IV; A-II A-II A-II; A-IV A-IV A-IV; A-IV A-IV A-II; A-IV A-IV A-I.

Oligomerisation modules

According to an especially preferred embodiment of the invention, the heterologous moiety is an oligomerising module. In this context, an oligomerising module is a peptide or a protein or part of a protein which is capable of interacting with other, similar or identical oligomerising modules. The interaction is of the type that

produces multimeric proteins or polypeptides. Such an interaction may be caused by covalent bonds between the components of the multimer as well as by hydrogen bond forces, hydrophobic forces, van der Waals forces, salt bridges. The invention also encompasses oligomerising modules of non-peptide nature such as a nucleic acid sequence of DNA, RNA, LNA, or PNA.

The oligomerisation module may be a dimerising module, a trimerising module, a tetramerising module, or a multimerising module.

When the apolipoprotein or analogue part of the construct is coupled to an oligomerising module, multimers of the construct can be made by simply mixing a solution of constructs (oligomerisation module linked to apolipoprotein part) under appropriate conditions. In this way, dimers, trimers, tretramers, pentamers, hexamers or higher –mers can be made depending on the type of oligomerising module being linked to the apolipoprotein part of the construct.

The multimers according to the invention may be homomers or heteromers, since different apolipoproteins can be linked to the oligomerising modules and be incorporated into the multimer. It may be advantageous to mix the different types of apolipoproteins in this way to obtain an improved clinical effect of the construct. Preferred homomers include trimers of Apo-A-I and trimers of Apo-A-IV.

According to an especially preferred embodiment of the invention the oligomerising module is from tetranectin and more specifically comprises the tetranectin trimerising structural element (hereafter termed TTSE), which is described in detail in WO 98/56906. The trimerising effect of TTSE is caused by a coiled coil structure which interacts with the coiled coil structure of two other TTSEs to form a trimer, which is exceptionally stable. A further advantage of TTSE is that it is a weak antigen (WO 98/56906).

Preferably the heparin binding site, which is located in the N-terminal region of exon 1 (Figure 4) is abolished by removal or mutagenis of N-terminal lysine residues (Graversen et al., manuscript) without inhibiting trimerisation. TTSEs that include most or all of exon 1 therefore confer an affinity for sulfated polysaccharides to any designed protein which encompasses such a TTSE as part of its structure. If desired, however, this affinity can be reduced or abolished by N-terminal truncation

or mutagenesis of lysine residues in the part of the TTSE that corresponds to the N-terminal 8-10 amino acid residues of exon 1 (Graversen et al., unpublished).

The interacting domain of the trimerising module according to the invention is preferably of the same type as in TTSE, namely a triple alpha helical coiled coil.

The TTSE may be from human tetranectin, from rabbit tetranectin, from murine tetranectin or from C-type lectin of shark cartilage. Preferably, the TTSE comprises a sequence having at least 68%, such as at least 75%, for example at least 81%, for example at least 87% such as at least 92% identity with the consensus sequence of figure 1. Thereby analogues of the TTSE having substantially the same trimerising effect are encompassed by the invention.

Preferably, the cystein residue 50 of TTSE should be mutagenised to serine, threonine, methionine or to any other amino acid residue in order to avoid formation of an unwanted inter-chain disulphide bridge, which could lead to unwanted multimerisation.

The presence of a trimer may be ascertained by well known techniques such as gel-filtration, SDS-PAGE, or native SDS gel electrophoresis depending on the nature of the trimer. One preferred method for ascertaining the presence of an oligomer is through linkage by DMSI followed by SDS-PAGE.

According to a preferred embodiment of the invention the protein construct is obtained by linking two or more apolipoproteins to oligomerising modules. The advantage of this embodiment is that the linkage of the individual apolipoproteins to one another does not take place within the apolipoprotein but in the oligomerising module. Thereby the nature of the wild-type apolipoprotein is conserved and the apolipoprotein conserves the secondary and tertiary structure, which is advantageous for its physiological function. By further introducing a peptide spacer between the apolipoprotein and the oligomerising module it is ensured that both of the components of the construct can perform their interaction with lipids and other oligomerising modules respectively without being affected by the interactions of the other component. Preferably, the peptide spacer is non-immunogenic, and has an essentially linear three dimensional structure.

Different or identical apo units may be oligomerised using an oligomerisation module such as a dimerising module, a trimerising module, a tetramerising module, a pentamerising module, a hexamerising module or a multimerising module. The oligomerising modules may comprise a coiled coil structure capable of interchain recognition and interaction.

The general method for producing an artificial oligomer of a protein or peptide comprises the identification of a trimerisation module from proteins that form trimers in nature. Through careful analysis, the domain responsible for the protein-protein interaction can be identified, isolated, and linked to the protein or peptide to be trimerised. According to the invention such trimerisation does not necessarily comprise the formation of a trimer of apolipoprotein or an analogue. It is also possible to link just one apolipoprotein to a trimerisation module and allow this peptide to trimerise with two other trimerisation modules. Thereby the molecular weight of the apolipoprotein part is increased and the plama half-life may be increased compared to native apolipoprotein.

One example of an oligomerisation module is disclosed in WO 95/31540 (HOPPE ET AL.), which describes polypeptides comprising a collectin neck region. The amino acid sequence constituting the collectin neck region may be attached to any polypeptide of choice. Trimers can then be made under appropriate conditions between three polypeptides comprising the collectin neck region amino acid sequence.

Spacer peptide

The protein construct may also advantageously comprise a spacer moiety, which is covalently linked between the apolipoprotein or apolipoprotein analogue and the heterologous moiety. The effect of the spacer is to provide space between the heterologous moiety and the apolipoprotein part of the construct. Thereby is ensured that the secondary structure of the apolipoprotein part is not affected by the presence of the heterologous moiety so that the physiological effect of the apolipoprotein part is maintained. Preferably, the spacer is of polypeptide nature. In this way the nucleic acid sequence encoding the spacer can be linked to the

sequence encoding the apolipoprotein part of the construct and optionally the sequence for the heterologous moiety, and the whole construct can be produced at the same time.

Design and preparation of suitable spacer moieties are known in the art and are conveniently effected by preparing fusion polypeptides having the format apo-spacer-X, where the spacer moiety is a polypeptide fragment (often a relatively inert one), so as to avoid undesired reactions between the spacer and the surroundings or the construct.

A spacer moiety may also be inserted between two TTSEs allowing both of these to interact with a third separate TTSE to form a trimeric complex, which then comprises two separate peptides: TTSE and TTSE-spacer-TTSE. This embodiment facilitates the production of the apolipoprotein construct since the major part of the trimer, which is then strictly seen a dimer, can be synthesised as one single polypeptide comprising in sequence (apo denoting any polypeptide sequence forming the apolipoprotein part of the construct) apo-TTSE-spacer-TTSE-apo.

In the embodiments where two TTSEs are present in the same monomer it is preferred that the spacer moiety has a length and a conformation which favours complex formation involving both of the two TTSEs which are covalently linked by the spacer moiety. In this way, problems arising from undesired formation of trimers of the formats (2+1+1), (2+2+2), and (2+2+1) (wherein only one TTSE of each monomer participates in complex formation) can be diminished.

The spacer peptide preferably comprises at least two amino acids, such as at least three amino acids, for example at least five amino acids, such as at least ten amino acids, for example at least 15 amino acids, such as at least 20 amino acids, for example at least 30 amino acids, such as at least 40 amino acids, for example at least 50 amino acids, such as at least 60 amino acids, for example at least 70 amino acids, such as at least 80 amino acids, such as at least 90 amino acids such as approximately 100 amino acids.

The spacer may be linked to the apo component and X through covalent linkages. and preferably the spacer is essentially non-immunogenic.

Similarly, the three-dimensional structure of the spacer is preferably linear or substantially linear.

The linkage

The two components of the construct may be linked together by a covalent linkage. This linkage may be formed between the component X and the C or N terminal amino acid of the apo component. The components may also be linked via more than one covalent linkages. The covalent linkage between the components may also comprise a S-S bridge, preferably between cystein residues. These cystein residues may be placed C or N terminally in the apo component and/or the component X or the may be placed internally in either or both of the components.

Carbohydrate

Irrespective of the other components of the construct the construct according to the invention may comprise a carbohydrate molety.

Tetranectin trimerising structural element

One especially preferred embodiment of the invention is the trimerisation or partial trimerisation of an apolipoprotein or analogue thereof with the trimerisation module from tetranectin.

This technique is described in WO 98/56906 (THØGERSEN ET AL.), which is hereby incorporated by reference. The trimeric polypeptides are constructed as a monomer polypeptide construct comprising at least one tetranectin trimerising structural element (TTSE), which is covalently linked to at least one heterologous molety. The tetranectin trimerising structural element is capable of forming a stable complex with two other tetranectin trimerising structural elements.

The term "trimerising structural element" (TTSE) used in the present description and claims is intended to refer to the portion of a polypeptide molecule of the tetranectin family which is responsible for trimerisation between monomers of the tetranectin

polypeptide. The term is also intended to embrace variants of a TTSE of a naturally occurring tetranectin family member, variants which have been modified in the amino acid sequence without adversely affecting, to any substantial degree, the trimerisation properties relative to those of the native tetranectin family member molecule.

Specific examples of such variants will be described in detail herein, but it is generally preferred that the TTSE is derived from human tetranectin, murine tetranectin, C-type lectin of human or bovine cartilage, or C-type lectin of shark cartilage. Especially preferred is monomer polypeptide constructs including at least one TTSE derived from human tetranectin.

The 49 residue polypeptide sequence encoded by exons 1 and 2 of tetranectin (Fig. 3) appears to be unique to the tetranectin group of proteins (Fig. 4) as no significant sequence homology to other known polypeptide sequences has been established. In preparation for experimental investigations of the architecture of tetranectin a collection of recombinant proteins was produced, the collection including complete tetranectin, the CRD domain (approximately corresponding to the polypeptide encoded by exon 3), a product corresponding to the polypeptide encoded by exons 2+3, a product corresponding to exons 1+2 (Holtet et al., 1996). Tetranectin is indeed a trimer, but the exon 2 encoded polypeptide is in fact capable of effecting trimerisation by itself as evidenced by the observation that the recombinant protein corresponding to exons 2+3 is in fact trimeric in solution.

3D-structure analysis of crystals of full-length recombinant tetranectin (Nielsen et al., 1996; Nielsen, 1996; Larsen et al., 1996; Kastrup, 1996) has shown that the polypeptide encoded in exon 2 plus three residues encoded in exon 3 form a triple alpha helical coiled coil structure.

From the combination of sequence and structure data it becomes clear that trimerisation in tetranectin is in fact generated by a structural element (Fig. 4), comprising the amino acid residues encoded by exon two and the first three residues of exon 3 by an unusual heptad repeat sequence, that apparently is unique to tetranectin and other members of its group: This amino acid sequence (Fig. 4) is characterised by two copies of heptad repeats (abcdefg) with hydrophobic residues

at a and d positions as are other alpha helical coiled coils. These two heptad repeats are in sequence followed by an unusual third copy of the heptad repeat, where glutamine 44 and glutamine 47 not only substitute the hydrophobic residues at both the a and d position, but are directly involved in the formation of the triple alpha helical coiled coil structure. These heptad repeats are additionally flanked by two half-repeats with hydrophobic residues at the d and a position, respectively.

The presence of beta-branched hydrophobic residues at a or d positions in alpha helical coiled coil are known to influence the state of oligomerisation. In the tetranectin structural element only one conserved valine (number 37) is present. At sequence position 29 in tetranectin no particular aliphatic residue appears to be preferred.

In summary, it is apparent that the triple stranded coiled coil structure in tetranectin to a large extent is governed by interactions that are unexpected in relation to those characteristic among the group of known coiled coil proteins.

The TTSEs form surprisingly stable trimeric molecules. The experimental observations, that (1) a substantial part of the recombinant proteins exists in the oligomeric state of and can be cross-linked as trimeric molecules even at 70C° and (2) that exchange of monomers between different trimers can only be detected after exposure to elevated temperature are evidence of a extremely high stability of the tetranectin trimerising structural element. This feature must be reflected in the amino acid sequence of the structural element. In particular, the presence and position of the glutamine containing repeat in the sequential array of heptad repeats is, together with the presence and relative position of the other conserved residues in the consensus sequence (Fig. 4), considered important for the formation of these stable trimeric molecules. For most practical uses the cysteine residue 50 should be mutagenized to serine, threonine, methionine or to any other amino acid residue in order to avoid formation of an unwanted inter-chain disulphide bridge, which eventually would lead to uncontrolled multimerisation, aggregation and precipitation of a polypeptide product harbouring this sequence.

In particular in conjunction with the trimer-stabilising exon 1 encoded polypeptide, the tetranectin trimerising structural element is a truly autonomous polypeptide

module retaining its structural integrity and propensity to generate a highly stabl homotrimeric complex whether it is attached or not by a peptide bond at either or at both termini to other proteins.

This unique property is demonstrated by the fact that polypeptide sequences derived from heterologous proteins may readily be trimerised when joined as fusion proteins to the tetranectin trimerising structural element. This remains valid irrespective of whether the heterologous polypeptide sequences are placed amino-terminally or carboxy-terminally to the trimerising element allowing for the formation of one molecular assembly containing up to six copies of one particular polypeptide sequence or functional entities, or the formation of one molecular assembly containing up to six different polypeptide sequences, each contributing their individual functional property.

Since three TTSEs of naturally occurring human tetranectin forms up a triple alpha helical coiled coil, it is preferred that the stable complex formed by the TTSEs of the invention also forms a triple alpha helical coiled coil.

The "tetranectin family" are polypeptides, which share the consensus sequence shown in Fig. 4 or a sequence, which is homologous at sequence level with this consensus sequence.

Hence, monomer polypeptide constructs of the invention are preferred which comprise a polypeptide sequence which has at least 68% sequence identity with the consensus sequence shown in Fig. 4, but higher sequence identities are preferred, such as at least 75%, at least 81%, at least 87%, and at least 92%.

Production of the protein construct

In order to produce a peptide component of the protein construct the cDNA encoding this part is inserted into an expression vector and transformed into a host cell.

The above mentioned host cell (which is also a part of the invention) can be prepared by traditional genetic engineering techniques which comprises inserting a

nucleic acid fragment (normally a DNA fragment) encoding the polypeptide part of a monomer polypeptide construct of the invention into a suitable expression vector, transforming a suitable host cell with the vector, and culturing the host cell under conditions allowing expression of the polypeptide part of the monomer polypeptide construct. The nucleic acid fragment encoding the polypeptide may be placed under the control of a suitable promoter which may be inducible or a constitutive promoter.

Depending on the expression system, the polypeptide may be recovered from the extracellular phase, the periplasm or from the cytoplasm of the host cell.

Suitable vector systems and host cells are well-known in the art as evidenced by the vast amount of literature and materials available to the skilled person. Since the present invention also relates to the use of the nucleic acid fragments of the invention in the construction of vectors and in host cells, the following provides a general discussion relating to such use and the particular considerations in practising this aspect of the invention.

In general, of course, prokaryotes are preferred for the initial cloning of nucleic sequences of the invention and constructing the vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example, strains such as E. coli K12 strain 294 (ATCC No. 31446), E. coli B, and E. coli X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes are also preferred for expression, since efficient purification and protein refolding strategies are available. The aforementioned strains, as well as E. coli W3110 (F-λ, prototrophic, ATCC No. 273325), bacilli such as Bacillus subtilis, or other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various Pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E.

coli species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.

The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilised, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in E. coli from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. Saccharomyces cerevislase, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980).

This plasmid already contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate

d hydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expres sion vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilisation. Any plasmid vector containing a yeast compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate in culture (tissue culture) has become a routine procedure (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the

HindIII site toward the BglI site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilise promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Upon production of the polypeptide monomer constructs it may be necessary to process the polypeptides further, e.g. by introducing non-proteinaceous functions in the polypeptide, by subjecting the material to suitable refolding conditions (e.g. by using the generally applicable strategies suggested in WO 94/18227), or by cleaving off undesired peptide moieties of the monomer (e.g. expression enhancing peptide fragments which are undesired in the end product).

In the light of the above discussion, the methods for recombinantly producing the monomer polypeptide construct of the invention are also a part of the invention, as are the vectors carrying and/or being capable of replicating the nucleic acids according to the invention in a host cell or a cell-line. According to the invention the expression vector can be e.g. a plasmid, a cosmid, a minichromosome, or a phage. Especially interesting are vectors which are integrated in the host cell/cell line genome after introduction in the host.

Another part of the invention are transformed cells (useful in the above-described methods) carrying and capable of replicating the nucleic acid fragments of the invention; the host cell can be a microorganism such as a bacterium, a yeast, or a protozoan, or a cell derived from a multicellular organism such as a fungus, an insect cell, a plant cell, or a mammalian cell. Especially interesting are cells from the bacterial species Escherichia, Bacillus and Salmonella, and a preferred bacterium is E. coli.

Yet another part of the invention relates to a stable cell line producing the

polypeptide part of a construct according to the invention, and preferably the cell line carries and expresses a nucleic acid of the invention.

Plasmids

The construct according to the invention may be manufactures using the plasmids disclosed below.

pT7H6 TripA-apoAI:

The plasmid comprises the plasmid pT7H6Fxtripa described in WO 98/56906 as example no. 1.

Expression is governed by the T7 promoter. The plasmid furthermore comprises a H6 sequence being a hexa-His affinity tag for use in purification. After that is inserted a Fxa recognition sequence (IQGR).

-SPGT is a connective sequence to the subsequent trimerisation module. This sequence has been inserted because it gives the opportunity to cut the DNA strand with Bgl II and Kpn I.

–Trip A is the trimerisation module from tetranectin..

GS is another connective sequence, which provides an opportunity to cut with Bam. HI.

Finally the plasmid comprises the human apoliprotein A-I cDNA coding for amino acids 25 -267 from human apolipoprotein A-I

pT7H6TripA-apoA1-del43:

The plasmid comprises the sequences as above, but the apolipoprotein part has been replaced with cDNA coding for amino acids 68-267 from human apolipoprotein A-I.

pT7H6UbiFxApoAI

The basic plasmid has been described in Ellgaard et al (1997).

The plasmid comprises the following sequences:

- the expression is governed by the T7 promoter

-H6: hexa-His affinity tag for purification of the protein construct

-Ubi: cDNA coding for human ubiquitin

-FX: recognition sequence for Fxa
-DNA coding for two Gly residues, necessary for the optimal cleavage by FXa.
-ApoAI: cDNA coding for amino acids 25 -267 from human apolipoprotein A-I

pT7H6UbiFXCysApoAI

As above, but after the sequence coding for the two glycine residues and before the apolipoprotein A-I sequence coding for a cystein residue has been inserted.

PT7H6FXCysApoAI

The plasmid comprises the following sequences:
- the expression is governed by the T7 promoter
-H6: hexa-His affinity tag for purification of the protein construct
-FX: recognition sequence for Fxa
-DNA coding for two Gly residues, necessary for the optimal cleavage by FXa.
-DNA coding for a cystein residue.
-ApoAI: cDNA coding for amino acids 25 -267 from human apolipoprotein A-I

Receptor binding

The performance of the constructs according to the invention may be analysed by measuring the ability of the constructs to bind to receptors which bind native apolipoprotein A-I, A-II or A-IV. Such receptors include but are not limited to cubilin and scavenger receptor B1. The dissociation constant, KD, of the complex between cubilin and native apolipoprotein A I is 20 nm. It has been determined experimentally that an apolipoprotein A I trimer according to the present invention binds even stronger to cubilin.

Affinity tags

The protein construct according to the invention may also comprise an affinity tag for use during purification of the construct. Such a tag preferably comprises a polyhistidine sequence. This sequence can advantageously be used for purification of the product on a Ni^{2+} column, which will bind the polyhistidine sequence and

thereby the whole protein. After lution from the column the polyhistidine sequence may be cleaved off by a proteinase such as trombin recognising a specific sequence built into the construct between the protein construct and the polyhistidine sequence.

In order to aid in the purification of the expressed protein a DNA sequence encoding an affinity tag may be added to the sequence encoding the protein construct. Such affinity tags may include well known tags as an antigenic tag, a polyhistidine tag, or a GST tag.

Production of apo-TTSE

In order to produce a construct comprising an apolipoprotein part and a TTSE, the cDNA encoding the apolipoprotein part is ligated at the 3' end to the 5' end of the c-DNA encoding the TTSE. Further TTSE units and apolipoprotein units may also be ligated. A sequence encoding an enzyme cleavage site is further ligated to the 3' end of the sequence encoding TTSE and finally a sequence encoding polyhistidine is also ligated. This can be done by conventional PCR techniques. The combined c-DNA is inserted into an expression vector and transformed into a host cell.

After expression in the E. coli, the polyhistidine sequence is used to capture the heterologous protein on a Ni2+ column. After elution the polyhistidine tail can be removed by a proteinase such as Fx cleaving the heterologous protein at the specific site inserted into it between the TTSE and the polyhistidine sequence. The resulting apo-TTSE peptide can then be processed further by trimerising it to other or identical apo-TTSE peptides.

Use of an apo construct for preparation of a pharmaceutical composition

The apo construct may be used for the preparation of a pharmaceutical composition. The composition may comprise pharmaceutical acceptable excipients, adjuvants, additives such as phospholipids, cholesterol, or triglycerides.

The pharmaceutical composition may be administered intravenously, intraarterially, intramusculary, transdermally, pulmonary, subcutaneously, intradermally,

intratechally, through the buccal-, anal-, vaginal-, conjunctival-, or intranasal tissue, or by inoculation into tissue, such as tumour tissue, or by an implant, or orally.

The formulation of the pharmaceutical compositions according to the invention is preferably performed using techniques well known to the skilled practitioner. This may comprise the addition of pharmaceutically acceptable excipients, adjuvants, or additives, such as phospholipids, cholestorol or triglycerides.

Administration of apo construct

The apo-constructs according to the invention may be administered for arteroscierosis such as for indications such as angina pectoris, claudication and for removal of endotoxins. It is envisaged that the administration comprises the administration of at least 50 mg of the construct every week such as to obtain a plasma concentration of approximately 0.5 g/L. Preferably the construct is administered parenterally such as through injections, suppositories, implants etc.

Preferably the composition is administered in an amount comprising at least 50 mg apolipoprotein construct per week, such as at least 100 mg/week, for example at least 250 mg/week, such as at least 500 mg/week, for example at least 750 mg/week such as at least 1000 mg/week, for example at least 1250 mg/week, such as at least 1500 mg/week, for example at least 2000 mg/week, such as at least 2500 mg/week, for example at least 5000 mg/week. The administration may be performed once a week once every second week, or once every third week, or once every fourth week.

The constructs may also be administered orally. For this administration route, the technology described in WO9946283, US 5,922,680, US 5,780,434 or US 5,591,433, US 5,609,871, or US 5,783,193 may be applied to the protein constructs according to the present invention. These references are hereby incorporated in their entirety by reference.

Cell population:

Th Invention also encompasses the use of the nucleotide sequence according to the invention for gene therapy.

The genes may be transferred to a population of macrophages and subsequently be transferred to the patient in need of treatment. Hereby, a transient expression of the gene is obtained, since the macrophage have a limited lifetime in the blood vessels.

Permanent transfection may be obtained by transforming liver cells.

Example 1: Cloning of Apo A-I

The cDNA encoding Apo A-I was amplified from a human liver cDNA library (Clontech) using standard PCR techniques. For the construction of Ubi-A-I the primers used were: 5'-CAC GGA TCC ATC GAG GGT AGG GGT GGA GAT GAA CCC CCC CAG AGC-3' and 5'- TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG-3'. The product was cloned into the vector pT7H6Ubi, described in (Ellgaard L. et al Eur. J. Biochem. 1997;244(2):544-51) using the Bam HI and Hind III cloning sites. For the construction of Trip-A-A-I the primers used were 5'-AAG GGA TCC GAT GAA CCC CCC CAG AGC CCC-3' and 5'-TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG-3'. The PCR product was cloned into the pT7H6tripa vector described in WO 98/56906 using the Bam HI and Hind III cloning sites. For the construction of Trip-A-I-del43 the primers used were 5'-AGG GGA TCC CTA AAG CTC CTT GAC AAC TGG G-3' and 5'- TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG -3'. The PCR product was cloned into the pT7H6tripa vector described in WO 98/56906 using the Bam HI and Hind III cloning sites. For the construction of Ubi-Cys-A-I the primers used were: 5'-GGT GGA TCC ATC GAG GGT AGG GGT GGA TGT GAT GAA CCC CCC C -3' and 5'- TCC AAG CTT ATT ACT GGG TGT TGA GCT TCT TAG TG -3'. The product was cloned inti the vector pT7H6Ubi, described in (Ellgaard L. et al Eur. J. Biochem. 1997;244(2):544-51) using the Bam HI and Hind III cloning sites. The plasmids generated are shown on figure 4, 5, 6, and 7.

Example 2: Expression of apolipoprotein A-I (apo A-I) in *E. coli*

Ubi-A-I and Trip-A-I are conveniently expressed in *E. coli* AV-1 cells (Stratag ne Inc.). Other cell lines may be used as well. Culturing of the cells and induction of expression were performed as described for tetranectin in WO 98/56906.

Example 3: Isolation and processing of protein

Crude protein was isolated by phenol extraction as described for tetranectin in WO 98/56906. The re-dissolved pellet from 6 litres of expression culture was centrifuged to remove non-dissolved material and then batch adsorbed to 50 ml Ni^{2+}-NTA-Sepharose, prepared as described in WO 98/56906. The column material was packed on a column and then washed with 500 ml 8 M urea, 500 mM NaCl, 50 mM Tris-HCl pH 8.0, then 200 ml of 6 M Guanidinium-HCl, 50 mM Tris-HCl pH 8.0 and finally 300 ml of 500 mM NaCl, 50 mM Tris-HCl pH 8.0. The protein was eluted with 500 mM NaCl, 50 mM Tris-HCl pH 8.0 and 10 mM EDTA. The protein was added 0.5 mg of FX_a and digested overnight at room temperature. Thrombin may be used for this purpose as well. The protein was gelfiltrated on a G-25 sephadex (Pharmacia) column in to a 500 mM NaCl, 50 mM Tris-HCl pH 8.0 buffer. Undigested protein was removed by passing the protein solution over a Ni^{2+}-NTA-Sepharose column pre-washed in 500 mM NaCl, 50 mM Tris-HCl pH 8.0 and then washed with 500 mM NaCl, 50 mM Tris-HCl pH 8.0. Undigested protein was eluted with 500 mM NaCl, 50 mM Tris-HCl pH 8.0 and 10 mM EDTA.

Example 4: Removal of lipids from the proteins

The proteins were gelfiltrated into a 10 mM $(NH_4)_2CO_3$ pH 8.8 solution and lyophilised. The lyophilised protein was resuspended in 25 ml cold 1:1 methanol/chloroform, incubated on ice for 30 min, centrifuged at 3000 *g* for 20 minutes. The pellet was resuspended in 25 ml of 1:2 cold methanol/chloroform, equilibrated for 30 minutes on ice and recentrifuged. The supernatant was removed and the pellet was briefly air-dried and then redissolved in 6 M guanidinium-HCl, 50 mM Tris-HCl pH 8.0 over night.

Example 5: Multimerisation assay

Cross linking

Multimerisation may be measured by cross-linking of multimers followed by analytical SDS-PAGE.

60 µl of a 0.2 mg/ml protein dissolved in 150 mM Na-borate pH 9.0 equillibrated to the desired temperature for 30 minutes are added 5 µl of a 20 mg/ml dimethylsuberimidate and incubated for 30 minutes at the desired temperature. The cross-linking was quenched by the addition of 5 µl 3 M Tris-HCl pH 9.0.

Dimethylsuberimidate causes lysin residues located within a short distance from one another to form a covalent bond. The result is that proteins which have formed multimers are covalently linked to one another. The molecular weight of the multimers can be estimated in the subsequent SDS-PAGE.

The cross-linking products were analysed by SDS-PAGE on 8-16 % acrylamide gels. Optionally an adjuvant, such as a lipid, was included in the cross-linking mixture, in which case the protein was pre-incubated with the adjuvant.

Analytical gelfiltration

Multimerisation may also be measured by analytical gelfiltration.

The protein was dissolved in a 500 mM NaCl, 50 mM Tris-HCl pH 8.0 buffer and gelfiltrated on a Superdex 200 HR 10/30 column in to the desired buffer at room temperature and a flow of 0.25 ml/min. For standard procedures the buffer was 100 mM NaCl, 50 mM Tris-HCl pH 8.0.

REFERENCES

Bergeron et al 1997, Biochem Biophys Acta, 1344:139-152.

Bolivar et al, 1977. Gene, 2: 95.

Chang et al. 1978. Nature, 275: 617-624.

Ellgaard et al (1997). Dissection of the domain architecture of the $\alpha_2$macroglobulin-receptor-associated protein. Eur J Biochem vol 244:544-551.

Fiers et al. 1978. Nature, 273: 113.

Goeddel et al. 1979. Nature, 281: 544.

Hess et al. 1969. Advances in Enzyme Regulation, 7: 149-166.

Hitzman et al. 1980. Journal of Biological Chemistry, 25: 12073-12080.

Holland et al. 1978. Biochemistry, 17: 4900.

Holtet, T.L., Graversen, J.H., Thøgersen, H.C. and Etzerodt, M. (1996).Domains and shared motifs in plasminogen - ligand interaction. Poster 21st Annual Lorne Conference on Protein Structure and Function, held Melbourne, Australia, February 4-8, 1996.

Itakura et al. 1977. Science, 198: 1056.

Jones. 1977. Genetics, 85: 23-33.

Kastrup, J.S. (1996). Lecture at Minisymposium held by EU HCM contract CHRX-CT93-0143: Protein Crystallography I in Hamburg, Germany, December 13-14, 1996.

Kingsman et al, 1979, Gene :141.

Larsen, I.K., Nielsen, B.B., Rasmussen, H. and Kastrup, J.S. (1996). Poster, 17th International Crystallography Congress, Seattle, USA held August 8-17. 1996.

Neame, P.J. and Boynton, R.E. (1996). Protein Soc. Symposium, (Meeting date 1995; 9th Meeting: Tech. Prot. Chem VII). Proceedings pp. 401-407 (Ed., Marshak, D.R.; Publisher: Academic, San Diego, Calif.).

Nielsen, B.B. (1996). Lecture, Lundbeck Centre Neuro-Medicinal Chemistry Minisymposium held November 5, 1996 at the Royal Danish School of Pharmacy, Copenhagen.

Nielsen, B.B., Larsen, I.K., Rasmussen, H. and Kastrup, J.S. (1996). Lecture, Danish Crystallographer's Meeting, held June 3-4, 1996 at the Royal Danish School of Pharmacy, Copenhagen.

Siebwenlist et al. 1980. Cell, 20: 269.

Sørensen et al, 1995, Gene, 152:243-245.

Stinchomb et al. 1979. Nature 282: 39.

Tschemper et al. 1980. Gene, 10: 157.

Claims

1. An apolipoprotein construct having the general formula

   - apo-X,

   - where apo is an apolipoprotein component selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, an analogue or a variant thereof,

   - and X is a heterologous moiety comprising at least one compound selected from the group consisting of an amino acid, a non-apolipoprotein peptide, an oligomerising module, a carbohydrate, a nucleic acid sequence, a non-apolipoprotein protein, and an apolipoprotein selected from the group consisting of apolipoprotein AI, apolipoprotein AII, apolipoprotein AIV, apolipoprotein E, an analogue or a variant thereof,

   - with the proviso that when the construct consists of exactly two identical, native apolipoproteins these are linked serially.

2. The construct of claim 1, further comprising a spacer between the apo component and X.

3. The construct of claim 2, wherein the spacer comprises a spacer peptide.

4. The construct of claim 3, wherein the spacer peptide comprises at least two amino acids, such as at least three amino acids, for example at least five amino acids, such as at least ten amino acids, for example at least 15 amino acids, such as at least 20 amino acids, for example at least 30 amino acids, such as at least 40 amino acids, for example at least 50 amino acids, such as at least 60 amino acids, for example at least 70 amino acids, such as at least 80 amino acids, such as at least 90 amino acids such as approximately 100 amino acids.

5. The construct according to claim 2, wherein the spacer is linked to the apo component and X through covalent linkages.

6. The construct according to claim 2, wherein the spacer is essentially non-immunogenic.

7. The construct according to claim 2, wherein the three-dimensional structure of the spacer is linear or substantially linear.

8. The construct of claim 1, wherein the component X is linked by a covalent link to the N-terminal or the C-terminal amino acid of apo.

9. The construct of claim 1, wherein the apo component and X are linked by two covalent links.

10. The construct of claim 1, wherein the apo component and X are linked through a S-S bridge, preferably a cystin bridge.

11. The construct of claim 1, wherein the protein comprises a non-immunogenic inert protein.

12. The construct of claim 11, wherein the protein comprises albumin.

13. The construct of claim 1, wherein the analogue or variant is capable of eliciting substantially the same physiological response as the apolipoprotein-A-I, A-II or A-IV.

14. The construct of claim 1, wherein the amino acid constituting the component X is a cystein residue.

15. The construct of claim 14, wherein the cystein residue is linked N-terminally, C-terminally, or internally to the apo component.

16. The construct of claim 1, wherein the peptide constituting the component X comprises more than 1 amino acids such as more than 2 amino acids, for example more than 5 amino acids, such as more than 10 amino acids, for example more than 15 amino acids, such as more than 20 amino acids, such as

more than 30 amino acids, for example more than 40 amino acids, such as more than 50 amino acids, for example more than 75 amino acids, such as more than 100 amino acids, for example more than 200 amino acids, such as more than 300 amino acids, for example more than 400 amino acids, such as more than 500 amino acids, for example more than 600 amino acids, such as more than 700 amino acids, for example more than 800 amino acids, such as more than 900 amino acids, for example more than 1000, 1250, 1500, 2000, or 2500 amino acids..

17. The construct of claim 1, wherein the protein comprised in the component X comprises two proteins selected from the group consisting of apolipoprotein A-I, apolipoprotein A-II, or apolipoprotein A-IV, an analogue or variant thereof.

18. The construct of claim 1, wherein the protein comprised in the component X comprises at least one apolipoprotein E.

19. The construct of claim 1, wherein the oligomerising module is a dimerising module.

20. The construct of claim 1, wherein the oligomerising module is a trimerising module.

21. The construct of claim 1, wherein the oligomerising module is a tetramerising modul.

22. The construct of claim 1, wherein the oligomerising module is a multimerising module.

23. The construct of claim 1, wherein the oligomerising module is of non-peptide nature.

24. The construct of claim 20, wherein the trimerising module comprises an amino acid sequence, capable of mediating interchain recognition, trimerisation and alignment of three polypeptide chains.

25. The construct of claim 20, wherein the trimerising module is from tetranectin.

26. The construct of claim 20, wherein the trimerising module comprises the tetranectin trimerising module from tetranectin.

27. The construct of claim 26, wherein the tetranectin trimerising module is capable of forming a stable complex with other tetranectin trimerising modules.

28. The construct of the claims 27, wherein the stable complex includes a coiled coil structure.

29. The construct of claim 28, wherein the coiled coil structure is a triple alpha helical coiled coil.

30. The construct of the claims 24 to 29, wherein the trimerising module comprising two tetranectin trimerising modules linked by a spacer moiety, which allows both of the two tetranectin trimerising modules to take part in a complex formation with a third tetranectin trimerising module not being part of the apolipoprotein construct.

31. The construct of the claims 24 to 30, wherein at least one tetranectin trimerising module is selected from the group consisting of human tetranectin, murine tetranectin or C-type lectin of human, bovine or shark cartilage.

32. The construct of the claims 24 to 31, wherein the tetranectin trimerising module comprises a sequence having at least 68 % identity with the consensus sequence of Fig. 4.

33. The construct of claim 32, wherein the sequence identity with the consensus sequence is at least 75 %, such as at least 81 %, for example at least 87 %, such as at least 92 %.

34. The construct of claim 32, wherein the cystein residue no. 50 is substituted by a serine residue, a threonine residue, or a methionine residue.

35. Th construct of claim 32, wherein the cystein residue no. 50 is substituted by any other amino acid residue.

36. The construct of claim 20, comprising the trimerisation module from the collectin neck region.

37. The construct of claim 1, wherein the oligomerising module is present after purification of the apolipoprotein construct.

38. The construct of claim 1, further comprising at least one carbohydrate moiety.

39. The construct of claim 1, further comprising an affinity tag.

40. The construct of claim 39, comprising a polyHis affinity tag.

41. The construct of claim 39, comprising an affinity tag selected from the group consisting of an antigenic tag, a GST tag.

42. The construct of claim 1, having a half-life of at least the half-life of native Apo A-I, A-II or A-IV, preferably at least 2 times higher, more preferably at least 3 times higher such as 4 times, more preferably at least 5 times higher, such as 6 times, more preferably at least 8 times higher such as at least 10 times.

43. The construct of claim 1, having a higher binding affinity to cholesterol compared to native Apo A-I, A-II or A-IV.

44. The construct of claim 1, capable of binding to cubilin.

45. The construct of claim 1, capable of binding to the scavenger receptor type B1.

46. The construct of claim 1, causing substantially no immunoresponse in humans.

47. The construct of claim 1, wherein the nucleic acid sequence comprises a DNA, a RNA, a PNA, or a LNA sequence.

48. Nucleic acid comprising a sequence of nucleotides encoding an apolipoprotein construct as defined in claim 1 to 47.

49. The nucleic acid of claim 48, wherein the encoding sequence is operably linked to a regulatory sequence for expression of the protein construct.

50. A vector comprising the nucleic acid of the claims 48 or 49.

51. A transformed host cell, comprising a nucleic acid sequence as defined in the claims 48 or 49.

52. A method for the production of an apolipoprotein construct as defined in the claims 1-47, comprising the steps of:

- culturing a transformed host cell under conditions promoting the expression of a protein construct according to claims 1 to 47,
- obtaining and recovering said protein construct,
- optionally, further processing said protein construct.

53. A method for the production of an apolipoprotein construct as defined in the claims 1-47, comprising the steps of:

- chemically synthesizing at least one oligomerising module, and subsequently
- linking said module to at least one apolipoprotein, an apolipoprotein analogue or an apolipoprotein variant,
- obtaining an apolipoprotein construct,
- isolating the resulting apolipoprotein construct,
- optionally, further processing said construct.

54. A method for the production of an apolipoprotein construct as defined in the claims 1-47, comprising the steps of:

- culturing a transformed host cell under conditions promoting the expression of an apolipoprotein or an apolipoprotein analogue or an apolipoprotein variant encoded by a nucleic acid fragment, and subsequently

- covalently linking said apolipoprotein or apolipoprotein analogue or apolipoprotein variant to a heterologous moiety,

- obtaining an apolipoprotein construct,

- isolating the resulting apolipoprotein construct,

- optionally, further processing said construct.

55. A method for the production of an apolipoprotein construct as defined in the claims 1-47, comprising the steps of:

- culturing a transformed host cell under conditions promoting the expression of a oligomerising module encoded by a nucleic acid fragment, and subsequently

- covalent linking said module to at least one apolipoprotein, an apolipoprotein analogue or an apolipoprotein variant,

- obtaining an apolipoprotein construct,

- isolating the resulting apolipoprotein construct,

- optionally, further processing said construct.

56. A pharmaceutical composition, comprising the apolipoprotein construct as defined in the claims 1-47.

57. The pharmaceutical composition of claim 56 adapted to be injected.

58. Use of an apolipoprotein construct as defined in the claims 1-47.

59. Use of an apolipoprotein construct as defined in the claims 1 to 47 for the preparation of a pharmaceutical composition.

60. The use of claim 59, wherein the pharmaceutical composition further comprises pharmaceutical acceptable excipients, adjuvants, additives, such as phospholipids, cholesterol, or triglycerides.

61. The use of claim 59, wherein the pharmaceutical composition is administered intravenously, intraarterially, intramusculary, transdermally, pulmonary, subcutaneously, intradermally, intratechally, through the buccal-, anal-, vaginal-, conjunctival-, or intranasal tissue, or by inoculation into tissue, such as tumour tissue, or by an implant, or orally.

62. The use of claim 59, comprising administering to an individual a composition comprising at least 50 mg apolipoprotein construct per week.

63. The use of claim 59, comprising administering to an individual a composition comprising at least at least 100 mg/week, for example at least 250 mg/week, such as at least 500 mg/week, for example at least 750 mg/week such as at least 1000 mg/week, for example at least 1250 mg/week, such as at least 1500 mg/week, for example at least 2000 mg/week, such as at least 2500 mg/week, for example at least 5000 mg/week.

64. The use of claim 59, comprising administering a dose of a pharmaceutical composition once a week.

65. The use of claim 59, comprising administering a dose of a pharmaceutical composition once every second week, or once every third week, or once every fourth week.

66. The use of claim 59, for the treatment and/or prevention of aterosclerosis.

67. The use of claim 59, in the treatment of neutralising endotoxins.

68. The use of claim 59, in the treatment of angina pectoris.

69. The use of claim 59, in the treatment of claudicatio.

70. Use of the nucleic acid sequence as defined in the claims 48 to 49 for gene therapy, wherein the DNA sequence encoding said apolipoprotein construct is used for transfection or infection of at least one cell population.

71. The use of claim 70, wherein the at least one cell population comprises macrophages.

72. The use of claim 70, wherein the at least one cell population comprises liver cells.

FIGURE 1

SQ SEQUENCE 267 AA; 30778 MW; 1A28B8366B620310 CRC64;

MKAAVLTLAV LFLTGSQARH FWQQDEPPQS PWDRVKDLAT VYVDVLKDSG RDYVSQFEGS

ALGKQLNLKL LDNWDSVTST FSKLREQLGP VTQEFWDNLE KETEGLRQEM SKDLEEVKAK

VQPYLDDFQK KWQEEMELYR QKVEPLRAEL QEGARQKLHE LQEKLSPLGE EMRDRARAHV

DALRTHLAPY SDELRQRLAA RLEALKENGG ARLAEYHAKA TEHLSTLSEK AKPALEDLRQ

GLLPVLESFK VSFLSALEEY TKKLNTQ

FIGURE 2

```
sp|P06727|APA4_HUMAN      MFLKAVVLTLALVAVAGARAEVSADQVATVMWDYFSQLSNNAKEAVEHLQKSELTQQLNA 60
sp|P33621|APA4_MACFA      MFLKAVVLTLALVAVTGARAEVSADQVATVMWDYFSQLSSNAKEAVEHLQKSELTQQLNA 60
sp|P06728|APA4_MOUSE      MFLKAAVLTLALVAITGTRAEVTSDQVANVVWDYFTQLSNNAKEAVEQFQKTDVTQQLST 60
sp|Q28758|APA4_PAPAN      ----------------GARAEVSADQVATVMWDYFSQLSSNAKEAVEHLQKSELTQQLNA 44
sp|O46409|APA4_PIG        MFLKAVVLSLALVAVTGARAEVNADQVATVMWDYFSQLGSNAKKAVEHLQKSELTQQLNT 60
sp|P02651|APA4_RAT        MFLKAVVLTVALVAITGTQAEVTSDQVANVMWDYFTQLSNNAKEAVEQLQKTDVTQQLNT 60
                                  *:.***.:****.*:****:**..***:***::**:::****.:

sp|P06727|APA4_HUMAN      LPQDKLGEVNTYAGDLQKKLVPFATELHERLAKDSEKLKEEIGKELEELRARLLPHANEV 120
sp|P33621|APA4_MACFA      LPQDKLGEVNTYAGDLQKKLVPFATELHERLAKDSEKLKEEIRKELEEVRARLLPHANEV 120
sp|P06728|APA4_MOUSE      LPQDKLGDASTYADGVHNKLVPFVVQLSGHLAKETERVKEEIKKELEDLRDRMMPHANKV 120
sp|Q28758|APA4_PAPAN      LPQDKLGEVNTYAGDLQKKLVPFATELHERLAKDSKKLKEEIRKELEEVRARLLPHANEV 104
sp|O46409|APA4_PIG        LPQDKLGEVNTYTEDLQKKLVPFATELHERLTKDSEKLKEEIRRELEELRARLLPHATEV 120
sp|P02651|APA4_RAT        LPQDKLGNINTYADDLQNKLVPFAVQLSGHLTKETERVREEIQKELEDLRANMMPHANKV 120
                          *******: .**: .:::*****..:*  :*:*:::::;*** :***:;* .::***.:*

sp|P06727|APA4_HUMAN      SQKIGDNLRELQQRLEPYADQLRTQVNTQAEQLRRQLTPYAQRMERVLRENADSLQASLR 180
sp|P33621|APA4_MACFA      SQKIGENVRELQQRLEPYTDQLRTQVNTQTEQLRRQLTPYAQRMERVLRENADSLQTSLR 180
sp|P06728|APA4_MOUSE      TQTFGENMQKLQEHLKPYAVDLQDQINTQTQEMKLQLTPYIQRMQTTIKENVDNLHTSMM 180
sp|Q28758|APA4_PAPAN      SQKIGENVRELQQRLEPYTDQLRTQVNTQTEQLRRQLTPYAQRMERVLRENADSLQTSLR 164
sp|O46409|APA4_PIG        SQKIGDNVRELQQRLGPFTGGLRTQVNTQVQQLQRQLKPYAERMESVLRQNIRNLEASVA 180
sp|P02651|APA4_RAT        SQMFGDNVQKLQEHLRPYATDLQAQINAQTQDMKRQLTPYIQRMQTTIQDNVENLQSSMV 180
                          :* :*:*:::*:**;:* *:: *: *:*:*:.:::: **.** :**: .:::* .*.:*:

sp|P06727|APA4_HUMAN      PHADELKAKIDQNVEELKGRLTPYADEFKVKIDQTVEELRRSLAPYAQDTQEKLNHQLEG 240
sp|P33621|APA4_MACFA      VHADQLKAKIDQNVEELKERLTPYADEFKVKIDQTVEELRRSLAPYAQDAQEKLNHQLEG 240
sp|P06728|APA4_MOUSE      PLATNLKDKFNRNMEELKGHLTPRANELKATIDQNLEDLRRSLAPLTVGVQEKLNHQMEG 240
sp|Q28758|APA4_PAPAN      PHADQLKAKIDQNVEELKGRLTPYADEFKVKIDQTVEELRRSLAPYAQDAQEKLNHQLEG 224
sp|O46409|APA4_PIG        PYADEFKAKIDQNVEELKGSLTPYAEELKAKIDQNVEELRRSLAPYAQDVQEKLNHQLEG 240
sp|P02651|APA4_RAT        PFANELKEKFNQNMEGLKGQLTPRANELKATIDQNLEDLRSRLAPLAEGVQEKLNHQMEG 240
                          * * ::* *;;;*;* **  *** *;*:*..***.:*:**  *** ; ..*******;**

sp|P06727|APA4_HUMAN      LTPQMKKNAEELKARISASAEELRQRLAPLAEDVRGNLKGNTEGLQKSLAELGGHLDQQV 300
sp|P33621|APA4_MACFA      LAPQMKKNAEELKARISASAEELRQRLAPLAEDMRGNLRGNTEGLQKSLAELGGHLDRHV 300
sp|P06728|APA4_MOUSE      LAPQMKKNAEELQTKVSAKIDQLQKNLAPLVEDVQSKVKGNTEGLQKSLEDLNRQLEQQV 300
sp|Q28758|APA4_PAPAN      LAPQMKKNAEELKARISASAEELRQRLAPLAEDMRGNLRGNTEGLQKSLAELGGHLDRHV 284
sp|O46409|APA4_PIG        LAPQMKKQAEELKAKISANADELRQKLVPVAENVHGHLKGNTEGLQKSLLELRSHLDQQV 300
sp|P02651|APA4_RAT        LAPQMKKNAEELQTKVSTNIDQLQKNLAPLVEDVQSKLKGNTEGLQKSLEDLNKQLDQQV 300
                          *:*****;****;;;;*:. ::*;;.*.*:.*;;;.;;::********** :*  :*;;;*

sp|P06727|APA4_HUMAN      EEFRRRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDKVNSFFSTFKE 360
sp|P33621|APA4_MACFA      EEFRLRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDKVNSFFSTFKE 360
sp|P06728|APA4_MOUSE      EEFRRTVEPMGEMFNKALVQQLEQFRQQLGPNSGEVESHLSFLEKSLREKVNSFMSTLEK 360
sp|Q28758|APA4_PAPAN      EEFRLRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDKVNSFFSTFKE 344
sp|O46409|APA4_PIG        EEFRLKVEPYGETFNKALVQQVEDLRQKLGPLAGDVEGHLSFLEKDLRDKVNTFFSTLKE 360
sp|P02651|APA4_RAT        EVFRRAVEPLGDKFNMALVQQMEKFRQQLGSDSGDVESHLSFLEKNLREKVSSFMSTLQK 360
                          * **  *** *; ** *****:*.;**:**. :*;**.*******.**;**.:*:**;;;

sp|P06727|APA4_HUMAN      KESQDKTLSLPEL----EQQQEQQQ----------------------------------EQ 383
sp|P33621|APA4_MACFA      KESQDNTLSLPEP----EQQREQQQEQQQEQEQEQQQQQSQQQQQEQQREQQQQEQQQEQ 416
sp|P06728|APA4_MOUSE      KGSPDQPQALPLPEQAQEQAQEQAQEQVQ------------------------------- 389
sp|Q28758|APA4_PAPAN      KESQDNTLSLPEP----EQQQEQQQEQEQ-----QQEQQEEQQQQEQQ-------QEQEQ 388
sp|O46409|APA4_PIG        EASQGQSQALPAQ----EKAQ--------------------------------------- 377
sp|P02651|APA4_RAT        KGSPDQPLALPLP----EQVQEQVQEQVQ------------------------------- 385
                          : * .:. :**      *: :

sp|P06727|APA4_HUMAN      QQEQVQMLAPLES 396
sp|P33621|APA4_MACFA      QQEQVQMLAPLES 429
sp|P06728|APA4_MOUSE      -------PKPLES 395
sp|Q28758|APA4_PAPAN      QQEQVQMLAPLES 401
sp|O46409|APA4_PIG        --------APLEG 382
sp|P02651|APA4_RAT        -------PKPLES 391
                             ***.
```

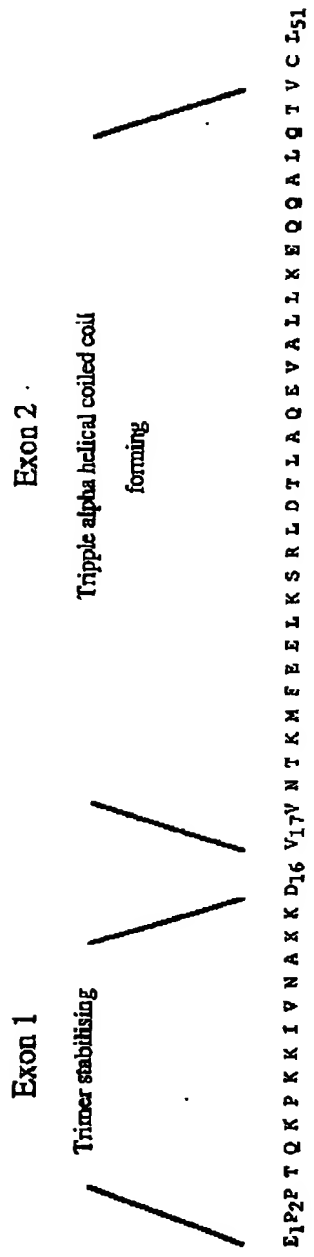


Figure 3

Position	d e f g a b c d e f g a b c d e f g a
Human tetranectin	V V N T K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T V C L K
Murine tetranectin	L V S S K M F E E L K N R M D V L A Q E V A L L K E K Q A L Q T V C L K
Bovine cart. protein	R R V K E K D G D L K T Q V E K L W R E V N A L K E M Q A L Q T V C L R
Shark cart. protein	S K S G K G K D D L R N E I D K L W R E V N S L K E M Q A L Q T V C L K
Consensus	L hy L E V L K E Q A L Q T V C L

Figure 4

FIGURE 5

pT7 H6UbiPx Apo A1

T7 promoter, H6, Ubiquitin and Apo A1:

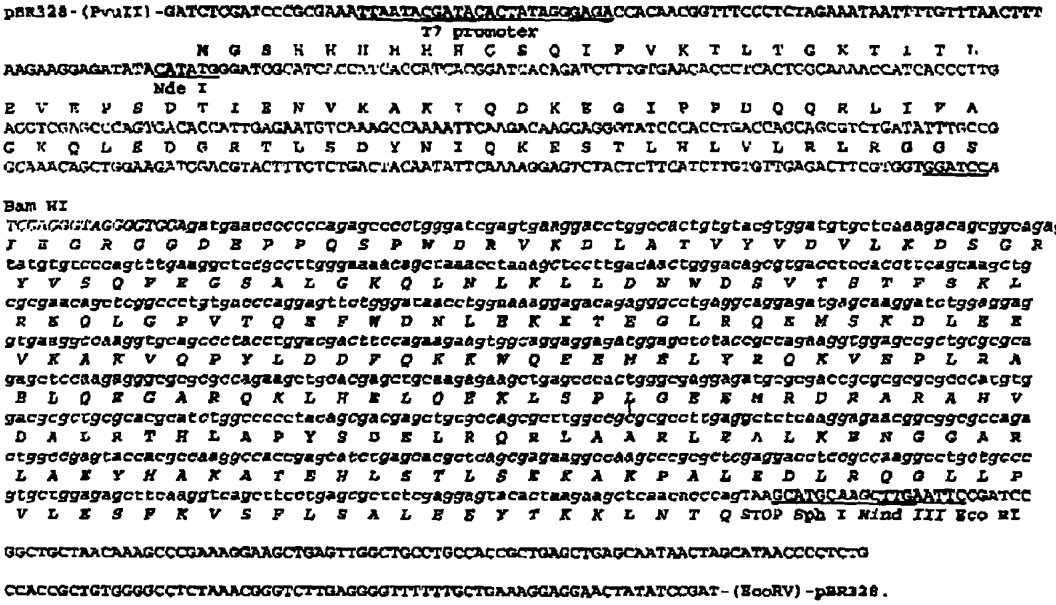


FIGURE 6

pT7 H6UbiPx Cys-Apo A1

T7 promoter, H6, Ubiquitin and Apo A1:

pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGATACACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT

T7 promoter

N G S H H H H H H G S Q I F V K T L T G K T I T L

AAGAAGGAGATATACATATGGGATCGCATCATCACCATCACGGATCACAGATCTTTGTGAAGACCCTCACTGGCAAAACCATCACCCTTG

Nde I

E V E P S D T I E N V K A K I Q D K E G I P P D Q Q R L I F A

AGGTCGAGCCCACTGACACATTGAGAATGTCAAAGCCAAAATTCAAGACAAGGAGGGTATCCCACCTGACCAGCAGCGTCTGATATTTGCCG

G K Q L E D G R T L S D Y N I Q K E S T L H L V L R L R G G S

GCAAACAGCTGGAAGATGGACGTACTTTGTCTGACTACAATATTCAAAAGCAGTCTACTCTTCATCTTGTGTTGAGACTTCGTGGTGGATCCA

Bam HI

TCGAGGCTAGGGGTGGATGTgatgaaccccccccagagccccctgggatcgagtgaaggacctggccactgtgtacgtggatgtgctcaaagacagcggcagagac

1 E C R G G C D E P P Q S P W D R V K D L A T V Y V D V L K D S G R D

catgtgtccccagtttgaaggctccgccttgggaaaacagctaaacctaaagctccttgacaactgggacagcgtgacctccaccttcagcaagctg

Y V S Q F E G S A L G K Q L N L K L L D N W D S V T S T F S K L

cgcgaacagccgggcccctgtgacccagggcgttctgggataacctggaaaaggagacagagggcctgaggcaggagatgagcaaggatctggaggag

R E Q L G P V T Q E F W D N L E K E T E G L R Q E M S K D L E E

gtgaaggccaaggtgcagccctacctggacgacttccagaagaagtggcaggaggagatggagctctaccgccagaaggtggagccgctgcgcgca

V K A K V Q P Y L D D F Q K K W Q E E M E L Y R Q K V E P L R A

gagccccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagcccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg

E L Q E G A R Q K L H E L Q E K L S P L G E E M R D R A R A H V

gacgcgctgcgcacgcatctggccccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaaggagaacggcggcgccaga

D A L R T H L A P Y S D E L R Q R L A A R L E A L K E N G G A R

ctggccgagtaccacgccaaggccaccgagcatctgagcacgctcagcgagaaggccaagcccgcgctcgaggacctccgccaagggctgctgccc

L A E Y H A K A T E H L S T L S E K A K P A L E D L R Q G L L P

gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccagTAAGCATGCAAGCTTGAATTCCGATCC

V L E S F K V S F L S A L E E Y T K K L N T Q SfOI Sph I Hind III Eco RI

GGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTTGG

CCACCGCTGTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.

FIGURE 7

pT7H6 Trip-A-Apo A1 - Amp^R.

T7 promoter, H6, FX$_a$-cleavage site and Trip-A insert sequence:

pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGATACACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGAT

T7 promoter

M G S H H H H H H G S I Q G R S P G T E P P T Q K P K K I V N A K K

ATACATATGGGATCGCATCACCATCACCATCACGGATCGATCCAGGGTACGTCTCCTGGTACCGAGCCACCAACCCAGAAGCCCAAGAAGATTGTAAATGCCAAGAAA

Bgl II Kpn I

D V V N T K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T V S L

GATGTTGTGAACACAAAGATGTTTGAGGAGCTCAAGAGCCGTCTGGACACCCTGGCCCAGGAGGTGGCCCTGCTGAAGGAGCAGCAGGCCCTGCAGACGGTCTCCCTG

Bam HI

AAGGGATCCgatgaaccccccagagccccctgggatcgagtgaaggacctggccactgtgtacgtggatgtgctcaaagacagcggcagagac

K G S *D E P P Q S P W D R V K D L A T V Y V D V L K D S G R D*

tatgtgtcccagtttgaaggctccgccttgggaaaacagctaaacctaaagctccttgacaactgggacagcgtgacctccaccttcagcaagctg

Y V S Q F E G S A L G K Q L N L K L L D N W D S V T S T F S K L

cgcgaacagctcggccctgtgacccaggagttctgggataacctggaaaaggagacagagggcctgaggcaggagatgagcaaggatctggaggag

R E Q L G P V T Q E F W D N L E K E T E G L R Q E M S K D L E E

gtgaaggccaaggtgcagccctacctggacgacttccagaagaagtggcaggaggagatggagctctaccgccagaaggtggagccgctgcgcgca

V K A K V Q P Y L D D F Q K K W Q E E M E L Y R Q K V E P L R A

gagctccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagcccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg

E L Q E G A R Q K L H E L Q E K L S P L G E E M R D R A R A H V

gacgcgctgcgcacgcatctggccccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaaggagaacggcggcgccaga

D A L R T H L A P Y S D E L R Q R L A A R L E A L K E N G G A R

ctggccgagtaccacgccaaggccaccgagcatctgagcacgctcagcgagaaggccaagcccgcgctcgaggacctccgccaaggcctgctgccc

L A E Y H A K A T E H L S T L S E K A K P A L E D L R Q G L L P

*gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccag*TAATAAGCTTGAATTCCGATCCGGCTGCTAA

V L E S F K V S F L S A L E E Y T K K L N T Q STOP Hind III Eco RI

CAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTCTGCCACCGCTGTGGGGCCTCTAAACGGGTCTTGAGGGG

TTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.

FIGURE 8

pT7H6 Trip-A-Apo A1-del43 - Amp^R.

T7 promoter, H6, FX$_a$-cleavage site and Trip-A insert sequence:

pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGATACACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGAT
T7 promoter
M G S H H H H H H G S I Q G R S P G T E P P T Q K P K K I V N A K K
ATACATATGGGATCGCATCACCATCACCATCACGGATCGATCCAGGGTAGATCTCCTGGTACCGAGCCACCAACCCAGAAGCCCAAGAAGATTGTAAATGCCAAGAAA
Bgl II Kpn I
D V V N T K M F E E L K S R L D T L A Q E V A L L K E Q Q A L Q T V S L
GATGTTGTGAACACAAAGATGTTTGAGGAGCTCAAGAGCCGTCTGGACACCCTGGCCCAGGAGGTGGCCCTGCTGAAGGAGCAGCAGGCCCTGCAGACGGTCTCCCTG
Bam HI
AAGGGATCCctaaagctccttgacaactgggacagcgtgacctccaccttcagcaagctg
K G S L K L L D N W D S V T S T F S K L

cgcgaacagctcggccctgtgacccaggagttctgggataacctggaaaaggagacagagggcctgaggcaggagatgagcaaggatctggaggag
R E Q L G P V T Q E F W D N L E K E T E G L R Q E M S K D L E E
gtgaaggccaaggtgcagccctacctggacgacttccagaagaagtggcaggaggagatggagctctaccgccagaaggtggagccgctgcgcgca
V K A K V Q P Y L D D F Q K K W Q E E M E L Y R Q K V E P L R A
gagctccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagcccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg
E L Q E G A R Q K L H E L Q E K L S P L G E E M R D R A R A H V
gacgcgctgcgcacgcatctggcccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaaggagaacggcggcgccaga
D A L R T H L A P Y S D E L R Q R L A A R L E A L K E N G G A R
ctggccgagtaccacgccaaggccaccgagcatctgagcacgctcagcgagaaggccaagcccgcgctcgaggacctccgccaaggcctgctgccc
L A E Y H A K A T E H L S T L S E K A K P A L E D L R Q G L L P
gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccagTAATAAGCTTGAATTCCGATCCGGCTGCTAA
V L E S F K V S F L S A L E E Y T K K L N T Q STOP Hind III Eco RI

CAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTCTGCCACCGCTGTGGGGCCTCTAAACGGGTCTTGAGGGG
TTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.

FIGURE 9

pT7 H6UbiFx Cys-Apo A1

T7 promoter, H6, Ubiquitin and Apo A1:

```
pBR328-(PvuII)-GATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT
                                T7 promoter
                  M  G  S  H  H  H  H  H  H  G  S  Q  I  F  V  K  T  L  T  G  K  T  I  T  L
AAGAAGGAGATATACATATGGGATCGCATCACCATCACCATCACGGATCACAGATCTTTCTGAAGACCCTCACTGGCAAAACCATCACCCTTG
                Nde I
E  V  E  P  S  D  T  I  E  N  V  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q  Q  R  L  I  F  A
AGGTCGACCCCAGTGACACCATTGAGAATGTCAAAGCCAAAATTCAAGACAAGGAGGGTATCCCACCTGACCAGCAGCGTCTGATATTTGCCG
G  K  Q  L  E  D  G  R  T  L  S  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G  S
GCAAACAGCTGGAAGATGGACGTACTTTGTCTGACTACAATATTCAAAAGGAGTCTACTCTTCATCTTGTGTTGAGACTTCGTGGTGGATCCA
                                                                                  Bam HI
TCGAGGTTAAGGGTGGATGTgatgaaccccccagagccccctgggatcgagtgaaggacctggccactgtgtacgtggatgtgctcaaagacagcggcagagac
A  E  G  E  G  G  C  D  E  P  P  Q  S  P  W  D  R  V  K  D  L  A  T  V  Y  V  D  V  L  K  D  S  G  R  D
tatgtgtccagtttgaaggctccgccttgggaaaacagctaaacctaaagctcttgacaactgggacagcgtgacctccaccttcagcaagctg
 Y  V  S  Q  F  E  G  S  A  L  G  K  Q  L  N  L  K  L  L  D  N  W  D  S  V  T  S  T  F  S  K  L
cgcgaacagctcggccctgtgacccaggagttctgggataaccctggaaaaggagacagagggcctgaggcaggagatgagcaaggatctggaggag
 R  E  Q  L  G  P  V  T  Q  E  F  W  D  N  L  E  K  E  T  E  G  L  R  Q  E  M  S  K  D  L  E  E
gtgaaggccaaggtgcagccctacctggacgacttccagaagaagtggcaggaggagatggagtctacgccagaaggtggagccgctgcgcgca
 V  K  A  K  V  Q  P  Y  L  D  D  F  Q  K  K  W  Q  E  E  M  E  L  Y  R  Q  K  V  E  P  L  R  A
gagctccaagagggcgcgcgccagaagctgcacgagctgcaagagaagctgagccccactgggcgaggagatgcgcgaccgcgcgcgcgcccatgtg
 E  L  Q  E  G  A  R  Q  K  L  H  E  L  Q  E  K  L  S  P  L  G  E  E  M  R  D  R  A  R  A  H  V
gacgcgctgcgcacgcatctggccccctacagcgacgagctgcgccagcgcttggccgcgcgccttgaggctctcaaggaggaacggcggcgccaga
 D  A  L  R  T  H  L  A  P  Y  S  D  E  L  R  Q  R  L  A  A  R  L  E  A  L  K  E  N  G  G  A  R
ctggccgagtaccacgccaaggccaccgagcatctgagcacgccagcgagaaggccaagcccgcgctgaggacctccgccaaggccctgctgccc
 L  A  E  Y  H  A  K  A  T  E  H  L  S  T  L  S  E  K  A  K  P  A  L  E  D  L  R  Q  G  L  L  P
gtgctggagagcttcaaggtcagcttcctgagcgctctcgaggagtacactaagaagctcaacacccagTAAGCATGCAAGCTTGAATTCCGATCC
 V  L  E  S  F  K  V  S  F  L  S  A  L  E  E  Y  T  K  K  L  N  T  Q STOP Sph I Hind III Eco RI

GGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCCTGCCACCGCTGAGCTGAGCAATAACTAGCATAACCCCTCTG

CCACCGCTGTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGAT-(EcoRV)-pBR328.
```

SEQUENCE LISTING

<110> ProteoPharma ApS

<120> Apolipoprotein

<130> Apolipoprotein

<140>
<141>

<160> 4

<170> PatentIn Ver. 2.1

<210> 1
<211> 243
<212> PRT
<213> Homo sapiens

<400> 1

```
Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala Thr
 1               5                  10                  15

Val Tyr Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln
            20                  25                  30

Phe Glu Gly Ser Ala Leu Gly Lys Gln Leu Asn Leu Lys Leu Leu Asp
        35                  40                  45

Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu
    50                  55                  60

Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu
65                  70                  75                  80

Gly Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala Lys
                85                  90                  95

Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu Met
            100                 105                 110

Glu Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln Glu
        115                 120                 125

Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro Leu
    130                 135                 140
```

Gly Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu Arg
145 150 155 160

Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala
165 170 175

Arg Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr
180 185 190

His Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala Lys
195 200 205

Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu Ser
210 215 220

Phe Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu
225 230 235 240

Asn Thr Gln

<210> 2
<211> 244
<212> PRT
<213> Homo sapiens

<400> 2
Cys Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala
1 5 10 15

Thr Val Tyr Val Asp Val Leu Lys Asp Ser Gly Arg Asp Tyr Val Ser
20 25 30

Gln Phe Glu Gly Ser Ala Leu Gly Lys Gln Leu Asn Leu Lys Leu Leu
35 40 45

Asp Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg Glu Gln
50 55 60

Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr
65 70 75 80

Glu Gly Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala
85 90 95

Lys Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu
100 105 110

Met Glu Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln
115 120 125

Glu Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro
130 135 140

Leu Gly Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu
145 150 155 160

Arg Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala
165 170 175

Ala Arg Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu
180 185 190

Tyr His Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala
195 200 205

Lys Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu
210 215 220

Ser Phe Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys
225 230 235 240

Leu Asn Thr Gln

<210> 3
<211> 301
<212> PRT
<213> Homo sapiens

<400> 3
Ser Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn
1 5 10 15

Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser
20 25 30

Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln
35 40 45

Ala Leu Gln Thr Val Ser Leu Lys Gly Ser Asp Glu Pro Pro Gln Ser
50 55 60

Pro Trp Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu

<213> Homo sapiens

<400> 4

```
Ser Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn
 1               5                  10                  15

Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser
            20                  25                  30

Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln
        35                  40                  45

Ala Leu Gln Thr Val Ser Leu Lys Gly Ser Leu Lys Leu Leu Asp Asn
    50                  55                  60

Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu Arg Glu Gln Leu Gly
65                  70                  75                  80

Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu Gly
                85                  90                  95

Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala Lys Val
            100                 105                 110

Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu Met Glu
        115                 120                 125

Leu Tyr Arg Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln Glu Gly
    130                 135                 140

Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys Leu Ser Pro Leu Gly
145                 150                 155                 160

Glu Glu Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu Arg Thr
                165                 170                 175

His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg
            180                 185                 190

Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His
        195                 200                 205

Ala Lys Ala Thr Glu His Leu Ser Thr Leu Ser Glu Lys Ala Lys Pro
    210                 215                 220

Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro Val Leu Glu Ser Phe
225                 230                 235                 240
```

5

Lys Val S r Phe Leu Ser Al Leu Glu Glu Tyr Thr Lys Lys Leu Asn
245 250 255

Thr Gln

US 0998710705P1

Creation date: 17-07-2003
Indexing Officer: JFUNSTEN - JAMES FUNSTEN
Team: OIPEBackFileIndexing
Dossier: 09987107

Legal Date: 12-02-2002

No.	Doccode	Number of pages
1	CRFL	6

Total number of pages: 6

Remarks:

Order of re-scan issued on .....